ETHANOL TOLERANCE IN YEASTS

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I. INTRODUCTION

Industries in which ethanol is the primary product of a *Saccharomyces* fermentation (i.e., breweries, wineries, distilleries, and sake producers) have evolved over a period of several thousand years. During that time, a general understanding arose that the maximum level of ethanol produced in each of these industries was a result of the intrinsic ability of the different strains of *Saccharomyces* used within each to tolerate ethanol. Thus, it was (and is) widely believed that sake yeasts, which produce "wines" of 20% (v/v) ethanol, are much more ethanol tolerant than brewers' yeasts which normally make beers of 4 to 5% (v/v) ethanol. The traditional and widely held order of ethanol tolerance in the genus *Saccharomyces* is sake yeasts > wine yeasts > distillers' yeasts > brewers' yeasts.

What, however, is "ethanol tolerance"? For a yeast property of such monumental importance, there has, until very recently, been little reported on the physiological nature of ethanol tolerance, its genetic regulation, how to define or assay it, the manner of excretion of ethanol from the cell, or the site(s) of ethanol toxicity. Only a few dozen reports on the subject were known until the mid-1970s. It was only with the advent of the energy crisis and replacement or supplementation of fossil fuels with ethanol-based products (e.g., gasohol) that ethanol tolerance in *Saccharomyces* yeast has received increased scientific attention.

The results of this research have been staggering. Not only are long-held fundamental beliefs on ethanol tolerance in *Saccharomyces* being challenged, but an appreciation has arisen on how wort preparation and fermentation conditions (quite different within each subindustry) profoundly influence the ethanol tolerance of the strain employed. At the same time, recent research has illustrated that ethanol tolerance in *Saccharomyces* yeast is not simply due to an intrinsic ability of each yeast to tolerate differing levels of ethanol. Instead, tolerance is dramatically influenced by wort nutritional conditions (especially with regards to unsaturated lipids and assimilable nitrogen), environmental parameters employed (especially temperature), and whether the carbohydrate substrate is added in a sequential manner (as in the sake industry) or is all present at the time of inoculation (as in brewing, distilling, and enology). Each of these factors has been shown over the past decade to play a large role in determining the maximum level of ethanol that a given *Saccharomyces* yeast can produce.

It is the primary objective of this review that the reader will develop a much greater appreciation of how the different practices of the alcohol-related industries played a major role in molding traditional beliefs on ethanol tolerance in *Saccharomyces* yeasts. Many other aspects of ethanol tolerance (especially as they apply to the production of

high concentrations of ethanol by brewers' yeasts — the emphasis of the authors' research program) will be examined. The current means to define and assay ethanol tolerance will be discussed and a great deal of attention will be given to exploring the considerable direct and indirect evidence which indicates the primary sites of ethanol toxicity in yeast. Reported effects of ethanol on yeast growth rate, fermentative rate, and viability will be reviewed, as will the most controversial areas of research into ethanol tolerance and metabolism in *Saccharomyces* yeasts, i.e., whether ethanol is transported by active transport or passive diffusion and whether or not *Saccharomyces* yeasts accumulate high concentrations of intracellular ethanol. These latter questions have only very recently been settled. Finally, an attempt will be made to summarize what is known today about ethanol tolerance in yeast and the many questions that remain to be answered.

II. ASSAYING AND DEFINING ETHANOL TOLERANCE

Universal and absolute levels of ethanol tolerance do not exist — primarily because of the lack of a widely accepted method for defining ethanol tolerance. Despite this, there is a general appreciation in industry that strains of *Saccharomyces cerevisiae* and *S. uvarum* differ in their ability to tolerate various levels of ethanol.[1] Strains used in brewing are stated to have only moderate ethanol tolerance[2] compared to those used in distilling.[3] Levels of 8 to 9% (v/v) ethanol are reported as the upper limits for brewers' yeasts.[4]

A. Tolerance Defined by the Effects of Ethanol on Batch Culture Growth

One of the most widely used methods to define ethanol tolerance is to determine the concentration of ethanol which will completely suppress batch growth. Because of its simplicity, it is an attractive test for screening large numbers of strains for their ability to tolerate ethanol.

An early example of this procedure is that of Ranganathan and Bhat.[5] In 1958, they evaluated the ethanol tolerance of 28 different yeast strains. Tolerance to ethanol was based on the ability to grow in 10 m*l* of a defined medium containing 1 to 14% (v/v) concentrations of ethanol. The highest ethanol level allowing growth after 48 hr at room temperature was considered to be the level of ethanol tolerance of that tested yeast. On the basis of this work, strains were subdivided into three categories of tolerance (poor, 3 to 6%; moderate, 6 to 10%; and high, 10 to 13%). No precautions were taken to prevent the evaporation of ethanol from the testing system. This has since been shown to affect the tolerance determination.[6]

More recently, in 1975, Day et al.[2] determined the growth of a variety of brewing, sake, and sugar-tolerant yeasts in synthetic medium and ale wort, with both containing added ethanol. Growth to a level of 10^6 cells per milliliter at 20°C, from a starting inoculum of 50 to 100 cells per milliliter, was scored as tolerance. The strains exhibited tolerances ranging from 7 to 13% (v/v) ethanol in synthetic medium and 10 to 13% in ale worts. Brewing strains were of intermediate tolerance, sake strains were very tolerant, and sugar-tolerant strains were relatively poor.

Rose[4] in 1980 and Inoue et al.[6] in 1962 defined ethanol tolerance in *Saccharomyces* yeasts as being the concentration of ethanol at which growth was just prevented. Studying sake yeast strains, Inoue et al.[6] found ethanol tolerances of 11 to 12.5% (v/v) in a medium containing 5% (w/v) glucose at 17°C. In addition, factors including temperature, ethanol losses through evaporation, substrate concentration, inoculum preparation, and nutrition were evaluated as to their influences on ethanol tolerance. Rose[4] approached the experiment in a similar fashion, using a defined glucose salts medium containing 1% (v/v) increments of ethanol. In his case, tolerance was defined as the

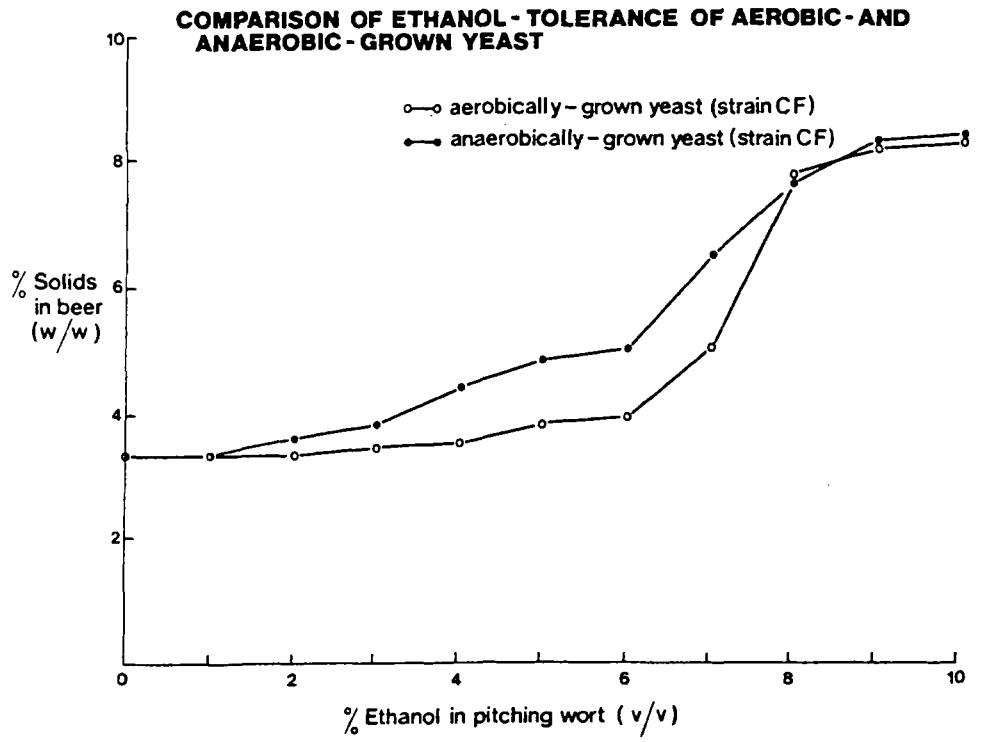

FIGURE 1. Comparison of ethanol tolerance of aerobically and anaerobically grown yeast. (From White, F. H., in *Proc. 15th Conv. Inst. Brew. (Aust. and N.Z. Sect.)*, Walter, L. S., Ed., Institute of Brewing, Sydney, 1978, 133. With permission.)

concentration of ethanol which completely suppressed growth after 72 hr at 30°C. Of four strains of *Saccharomyces* tested, a brewing strain had considerably less tolerance (7% [v/v] ethanol) than did three strains originating from distilleries and sake breweries (12 to 13% [v/v] ethanol).

White[7] in 1978 evaluated the ethanol tolerance of commercial ale and lager brewing yeasts. His system consisted of tube fermentors containing 10% (w/v) wort and initial ethanol levels ranging from 0 to 10% (v/v). Tolerance was defined as the ability to end-ferment the wort. In general, most strains tested were able to tolerate initial ethanol concentrations of 5 to 6% (v/v) without impairment of their ability to grow and convert wort carbohydrates. Ale yeasts *(S. cerevisiae)*, however, were slightly less tolerant than lager yeasts *(S. uvarum)*, as found by Day et al.[2] The previous history of the brewing yeast (especially whether it had been grown under aerobic or anaerobic conditions) and the nutritional condition of the wort (in particular that of its unsaturated lipid content) were also shown by White[7] to influence the ethanol tolerance of the yeast (Figures 1 and 2).

Variations of the above growth methods have been reported, with 12% (v/v) ethanol generally accepted as the limit for ethanol tolerance.[8,9]

B. Tolerance Defined by the Effects of Ethanol on Fermentative Ability

A second procedure utilized to define ethanol tolerance in *Saccharomyces* yeasts established the ratio of fermentative activity of a yeast culture in a medium in the absence of ethanol to the activity in the same medium containing a certain level of ethanol. For example, Nojiro and Ouchii[10] defined tolerance as the ratio of fermentative activity of yeast in a 2% (w/v) glucose medium in the absence of ethanol over that

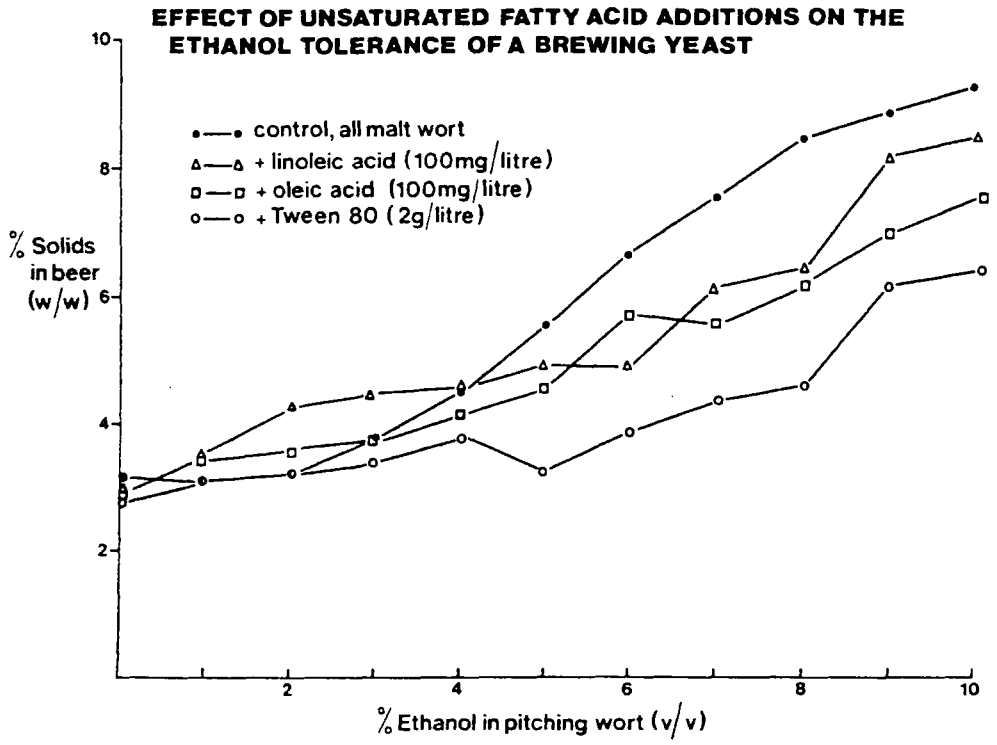

FIGURE 2. Effect of unsaturated fatty acid additions on the ethanol tolerance of a brewing yeast. (From White, F. H., in *Proc. 15th Conv. Inst. Brew. (Aust. and N.Z. Sect.)*, Walter, L. S., Ed., Institute of Brewing, Sydney, 1978, 133. With permission.)

in the same medium containing 18% (v/v) ethanol. Tolerances of 0.2 to 0.3 were found, with little difference seen between sake, brewers', wine, distillers', or bakers' strains of *Saccharomyces*. Other modifications of this approach have been described.[11-13]

The major disadvantage of these methods is that they do not directly express ethanol tolerance in terms of an absolute concentration of ethanol. Moreover, comparisons at a single ethanol concentration do not give a good indication of overall inhibition kinetics.

One method which does however state an absolute concentration of ethanol is that which defines tolerance as the concentration of ethanol at which fermentative activity completely ceases. The method assumes that substrate availability is nonlimiting, and ethanol tolerances of 13 and 14.5% (v/v) have been reported for *S. cerevisiae* PY-1 and UQM 73Y, respectively.[14] A more recently described method[15] reports IF_{50} values — the concentration of ethanol (v/v) required to inhibit the fermentative power of the yeast under test (in the absence of ethanol) by 50%.

C. Tolerance Defined by the Effects of Ethanol on Cell Viability

The rate of viability loss in the presence of ethanol has been used as a means to assess ethanol tolerance in a number of different yeast strains,[2,6,15] as well as to measure the influences of nutritional and environmental conditions on ethanol tolerance.[2,16-19] Losses in cell viability found in high-gravity brewery fermentations have been attributed to the killing effects of ethanol,[2,7] and it has been suggested that resistance to killing by ethanol may be related to ethanol tolerance.[2,6]

Inoue et al.[6] found that a yeast strain with high ethanol tolerance (based on the

Table 1
ATTENUATION AND VIABILITIES OF YEASTS IN WORT FERMENTATIONS OF 20.7° P

Yeast strain		Final gravity (°P)	Final viability (%)
Ale yeasts[a] *S. cerevisiae*	AB 1	5.3	67
	AB 80	5.6	89
	NCYC 1245	5.3	77
	NCYC 1026	5.3	83
	NCYC 240	5.6	74
Lager yeasts[a] *S. carlsbergensis*	AB 140	5.3	85
	AB 97	6.0	69
	NCYC 1324	5.6	89
Sake yeasts[b] *S. cerevisiae*	6	12.9	93
	NCYC 478	13.1	79
	CBS 1198	13.1	87

[a] ≈8% (w/v) ethanol (final).
[b] ≈4% (w/v) ethanol (final).

Adapted from Day, A., Anderson, E., and Martin, P. A., in *Proc. 15th Conv. Eur. Brew. Congr.*, IRL Press, Oxford, 1975, 377.

maximum level of ethanol which prevents growth) showed a slower rate of viability loss than a less tolerant strain when suspended in 13% (v/v) ethanol. To date, however, only one broad survey[2] has been carried out on the viability of different industrial yeast strains in the presence of moderate concentrations of produced ethanol (Table 1). Using 20.7° Plato (P) brewers' wort, Day et al.[2] found that brewers' yeasts were able to manufacture higher concentrations of ethanol than sake yeasts (approximately 8% [w/v] vs. 4%), but they had much lower viabilities by the end of the fermentations.

A selection procedure based on viability in the presence of ethanol has been suggested to be useful for identifying mutants with increased tolerance.[16] Selected mutants, derived from a continuous chemostat fermentation in the presence of ethanol, showed increased ethanol tolerance over that of the wild-type strains.[20] Increased rates of survival in 14% (w/v) ethanol have been used as a means to demonstrate improvements in ethanol tolerance related to the induction of heat shock proteins.[18,21]

Increased survival rates in the presence of ethanol can be brought about by changes in the nutritional composition of the growth medium. Thomas et al.[17] found that cells enriched in ergosterol and linoleic acid had increased survival rates in ethanol over cells enriched in the sterol with oleic acid. Day et al.[2] reported that the presence of oleic acid, pantothenate, or oxygenation during active growth increased the survival of an ale yeast suspended in 10% (v/v) ethanol.

Kalmokoff and Ingledew[15] compared the resistances to ethanol-induced losses in viability of an ale (*S. cerevisiae* NCYC 366), lager (*S. uvarum* NCYC 1324), bakers' *(S. cerevisiae)*, and sake (*S. sake* IFO 2347 Kyokai No. 7) yeast. For all yeasts, losses were exponential in nature (as seen in Figure 3 for *S. sake*), with early stationary phase cells showing a greater resistance towards the killing effect than cells in the midexponential phase. The minimal level of ethanol required to inhibit viability in stationary phase suspensions was 18, 18, 13, and 15% (v/v) for the sake, ale, lager, and bakers' yeast, respectively. Such concentrations were considerably above those at which growth was totally suppressed, suggesting that cell death is not a predominant factor

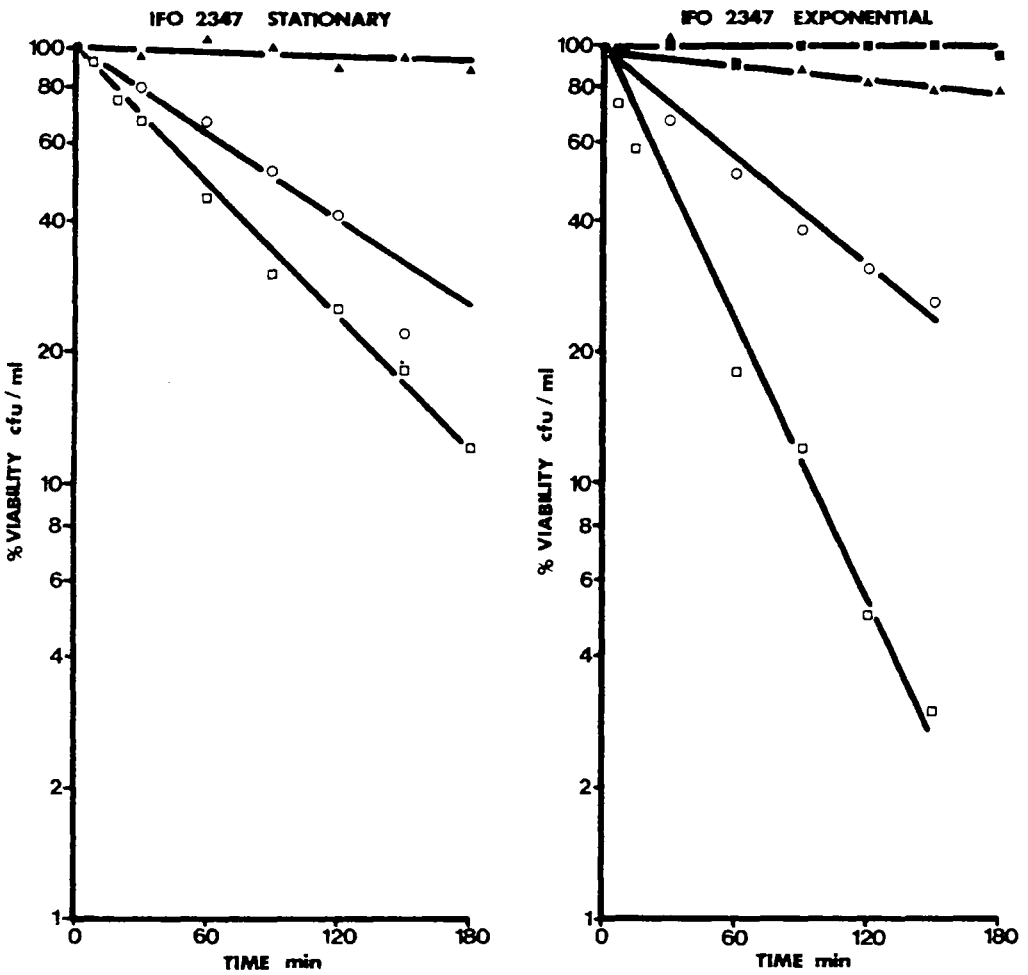

FIGURE 3. Decrease in viability (cfu/m*l*) of *S. sake* IFO 2347 over a 3-hr period for both midexponential and early stationary phase cell suspensions. ■, 13%; ▲, 15%; ○, 18%; □, 21% ethanol (v/v). (From Kalmokoff, M. L., Evaluation of Ethanol Tolerance in Selected *Saccharomyces* Strains, M.Sc. thesis, University of Saskatchewan, Saskatoon, 1985. With permission.)

in growth inhibition under the conditions employed.[15] In some circumstances, however, especially when high ethanol concentrations are combined with high temperatures, losses in viability can influence measurements of ethanol tolerances of both fermentative ability[16] and growth.[22]

D. Tolerance Defined as the Maximum Level of Produced Ethanol

The highest concentration of ethanol produced under defined conditions in batch culture or sake fermentations has been frequently used as a means to measure ethanol tolerance.[23-28]

In very high gravity brewing, ethanol production up to 16% (v/v) within normal brewing time periods has been reported by Casey and co-workers,[23,24,29,30] utilizing unmodified strains of commercial lager yeasts. Providing that the proper nutritional conditions were present, brewing yeasts under batch conditions were concluded to be tolerant to the same levels of ethanol as wine, distillers', and sake yeasts. Such ethanol levels are considerably higher than those reported by White,[7] who concluded brewers' yeasts to be tolerant to 9 to 11% (v/v) ethanol.

Table 2
MAXIMUM % ETHANOL (v/v) PRODUCED IN BOTH LIPID-SUPPLEMENTED AND NONSUPPLEMENTED CONDITIONS FOR EACH OF THE TESTED STRAINS

Strain	Maximum % ethanol (v/v)	
	Supplemented	Nonsupplemented
S. sake IFO 2347	5.5 (34)	3.2 (19)
S. cerevisiae NCYC 366	12.2 (74)	7.5 (51)
S. uvarum NCYC 1324	13.0 (85)	10.8 (75)
Bakers' yeast	13.3 (88)	11.3 (72)

Note: Numbers in parentheses are the percent of the fermentable sugar that was utilized.

From Kalmokoff, M. and Ingledew, W. M., *J. Am. Soc. Brew. Chem.*, 43(4), 189, 1985. With permission.

The ethanol tolerance of sake yeast[26,27] and related *Saccharomyces* yeasts isolated from sake fermentations[25] has been measured as the maximum amount of ethanol produced in a sake-type fermentation. Sake fermentations differ from batch fermentations (i.e., in brewing, distilling, and enology) in that the substrate is added in a stepwise fashion over very long fermentation times (in excess of 30 days), i.e., they are fed-batch fermentations.[31] By this method, the ethanol tolerance of *S. cerevisiae* var. *sake* Kyokai No. 7 was reported to be 20% (v/v) ethanol.[26] Flor and Hayashida[25] reported that a newly isolated yeast from sake fermentations, *S. uvarum* var. *inulyticus*, was capable of producing up to 22.4% (v/v) ethanol at 15°C in 35 days. In a sake-type fermentation with a brewers' strain of *S. cerevisiae*, Steinkraus et al.[272] reported the production of 25.6% ethanol. This represents the highest level of ethanol ever reported in the literature as produced by a *Saccharomyces* yeast.

However, to illustrate how definitions of ethanol tolerance can vary, Kalmokoff and Ingledew[15] determined the maximum level of ethanol that could be produced by an ale (*S. cerevisiae* NCYC 366), lager (*S. uvarum* NCYC 1324), bakers' *(S. cerevisiae)*, and sake *(S. sake* IFO 2347) yeast in a 30°P maltose-adjunct brewers' wort (Table 2). The sake yeast in this medium was considerably lower in tolerance. The ale, lager, and bakers' yeast all produced over 12% (v/v) ethanol in sterol/oleic acid-supplemented medium (also illustrating the importance of nutrition in defining tolerance), compared to only 5.5% (v/v) by the sake yeast. As the same sake strain can produce over 20% (v/v) ethanol in sake fermentations,[26] the medium employed here was in some way nutritionally limited and/or the yeast had poor resistance to the substrate effects on growth and fermentative ability. In this work, the high concentration of substrate was all present initially, rather than being added sequentially as in sake fermentations.

Other examples illustrating these priniciples, as well as casting doubt on there being major differences in ethanol tolerance between sake and brewers' yeast, exist in the literature. Traditionally, it has been felt that the sake yeast (selected for over centuries of sake production) had an inherently higher degree of ethanol tolerance than brewing yeast, with the latter considered to be of low ethanol tolerance.[4] Hayashida and Ohta[27] investigated the maximum level of ethanol that seven different strains of *Saccharomyces* could produce under the same conditions as sake fermentation. The fermentations were carried out at 20°C with the stepwise addition of substrate into a chemically defined medium with and without proteolipid (PL) (koji) supplement over very ex-

tended time periods (up to 150 days). They found that commercial brewing strains of *S. uvarum* and *S. cerevisiae* were able to produce 18.3 and 17.7% (v/v) ethanol, respectively, in basal medium and 20.3 and 19.9% (v/v), respectively, in koji-supplemented medium. It was concluded that the formation of high levels of ethanol was not a characteristic confined to the sake yeast, but arose due to conditions of the fermentation.[27]

The above work on sake fermentations and high gravity brewing indicates that inherent differences in tolerance between the yeasts employed in these industries are not extensive. Providing that optimal nutritional conditions are present, the production of high ethanol concentrations may be possible with any industrial *Saccharomyces* yeast.[15,30] Determinations of tolerance based on the maximum level of ethanol produced must therefore ensure that optimal conditions for maximum production are present. Otherwise, the ethanol tolerance will be a reflection of the process utilized rather than the inherent ability of the yeast to tolerate ethanol.

Species of *Saccharomyces* yeasts other than those employed in the alcohol-producing industries have been found to possess lower levels of ethanol tolerance. For example, Pierce et al.,[28] in a 27% (w/v) glucose medium (containing 5% [w/v] ethanol initially), reported tolerances of 13 to 14% (w/v) for *S. uvarum* 26602 and *S. cerevisiae* 26603, compared to only 8 to 11% (w/v) for *S. rouxii* ATCC 8383 and *S. bisporus* ATCC 28852. This occurred despite the fact that the latter two yeasts are more resistant to the effects of substrate inhibition.[28]

An additional factor affecting the maximum level of ethanol that a yeast can produce is temperature. In general, temperatures at the lower and upper ranges of those normally tolerated by a yeast will reduce the ability of the yeast to produce ethanol. These observations will, however, be discussed later in more detail.

III. MECHANISMS OF ETHANOL TOXICITY

A. Ethanol Inhibition of Growth and Fermentation Rates

1. Introduction

There is virtually universal agreement in the literature that ethanol inhibits growth and fermentation by *Saccharomyces* and other genera of yeast in a noncompetitive fashion.[15,16,20,32-64] By this, it is meant that ethanol affects the maximum specific rates of fermentation and growth, but not substrate affinity.

Some diversity of opinion on this matter has arisen when researchers have attempted to establish mathematical correlations between growth and fermentation rates in the absence and presence of ethanol and the actual ethanol concentration. Depending on the experimental data obtained, the exact manner of rate of inhibition has been reported to fit linear,[39,40,45,54,55,58] exponential,[20,32,33,43,46,47,50-52,60] hyperbolic,[16,32,38,49,53] or more complex models.[16,35] In part, these differences can be attributed to the wide diversity of yeast strains employed by different researchers. However, since initial substrate concentration, medium (nutritional) composition, and cultural and growth conditions can all influence the kinetics of ethanol inhibition, the profusion of models is not surprising.

2. Ethanol Inhibition of Growth Rate

One of the first measurable properties of yeast to be adversely affected by ethanol is that of growth rate. The specific growth rate is normally expressed by a Monod-type relationship as shown in Equation 1:

$$\mu = \mu_o \frac{S}{K_m + S} \quad (1)$$

Table 3
"THRESHOLD" CONCENTRATIONS OF ETHANOL DETERMINED FOR DIFFERENT *SACCHAROMYCES* YEASTS

Yeast strain employed	Threshold concentration of ethanol (% [w/v])	Ref.
S. uvarum NCYC 1324	2.0	15
S. cerevisiae ATCC 4126	2.5	35
S. cerevisiae var. *ellipsoideus*	2.6	40
S. cerevisiae NCYC 366	3.5	61
S. cerevisiae var. *sake* IFO 2347	3.2	15
S. cerevisiae NCYC 366	6.3	15

Note: The "threshold" concentration is the minimum concentration of ethanol required before any inhibition of the growth rate is observed.

where S is the concentration of substrate (gram per liter) μ_o is the maximum specific growth rate (1/hr) and K_m is the Monod constant. This equation, however, only holds true in the absence of toxic metabolic products. Ethanol, being toxic, decreases the value of the specific growth rate in *Saccharomyces* yeasts, with the mathematics of this decrease varying considerably. Sample relationships however are listed below in Equations 2 to 4.

Linear relationship[40] — Using *S. cerevisiae* var. *ellipsoideus*

$$\mu_i = \mu_{max}\left(1 - \frac{P_i}{P_m}\right) \quad (2)$$

Exponential relationship[33] — Using *S. cerevisiae* bakers' yeast

$$\mu_i = \mu_{max}(e^{-k_2 P_i}) \quad (3)$$

Hyperbolic relationship[53] — Using *S. cerevisiae* UG5

$$\mu_i = \mu_{max}\left(\frac{K_{IP}}{P_i + K_{IP}}\right) \quad (4)$$

where μ_i = specific growth rate (1/hr) in the presence of P_i concentration of ethanol, μ_{max} = maximum specific growth rate (1/hr) in the absence of ethanol, P_i = concentration of ethanol (gram per liter), P_m = ethanol concentration (gram per liter) above which growth will not occur, K_{IP} = product inhibition constant (gram per liter) on specific growth rate, and K_2 = empirical constant (gram per liter).

Two commonly reported values in studies on the inhibition of growth rate by ethanol are "threshold" concentrations of ethanol (defined as the minimum level of ethanol required before the growth rate is inhibited) and the "$\mu_{max} = 0$" concentration of ethanol (the concentration of ethanol at which the specific growth rate is equal to zero). As illustrated in Table 3, threshold concentrations normally fall within the 2 to 4% (w/v) ethanol range. Concentrations of ethanol at which $\mu_{max} = 0$ (Table 4) fall over a wider range, however, varying from 6.9 to 11.3% (w/v).

Table 4
CONCENTRATIONS OF ETHANOL REQUIRED FOR COMPLETE INHIBITION OF GROWTH FOR DIFFERENT *SACCHAROMYCES* YEASTS

Yeast strain employed	Ethanol concentration (% [w/v])	Ref.
Saccharomyces bakers' yeast	7.6	33
S. cerevisiae var. *ellipsoides*	6.9	40
S. cerevisiae ATCC 4126	9.3	35
S. uvarum ATCC 26602	9.5	57
S. cerevisiae NRRL-Y-132	8.7	39
S. cerevisiae bakers' yeast	7.0	63
S. cerevisiae bakers' yeast	11.3	44
S. cerevisiae UQM 70Y	10.0	20
S. cerevisiae UG5	9.6	53
S. sake IFO 2347	10.3	15
S. cerevisiae NCYC 366	9.5	15
S. uvarum NCYC 1324	9.4	15
S. cerevisiae bakers' yeast	9.5	15

In 1985, Kalmokoff and Ingledew,[15] using a 1.2% (w/v) maltose medium, compared the ethanol tolerance of four strains of *Saccharomyces* yeast expected to exhibit differences in ethanol tolerance: *S. cerevisiae* NCYC 366 (an ale yeast), *S. uvarum* NCYC 1324 (a lager yeast), *S. sake* IFO 2347 (sake yeast Kyokai No. 7), and *S. cerevisiae* (commercial bakers' yeast). Ethanol concentrations of 0 to 14% (v/v), in 2% increments, were tested. In all cases, above a linear threshold concentration, inhibition of growth by added ethanol exhibited linear inhibition kinetics (Figure 4). A surprising result, however, was that growth was completely suppressed in the four strains over a very narrow range — 11.8 to 13% (v/v) (calculated by extrapolation), suggesting similar levels of ethanol tolerance among the four yeasts as assayed by this method.

All of the above researchers have studied the effect of added ethanol on yeast growth rate, and in general, little inhibition was seen below 2% (w/v) ethanol (Table 3). However, ethanol inhibition of growth and fermentation rates occurs at much lower concentrations of ethanol if the yeast produces it endogenously. Added ethanol, for reasons not yet clearly understood, is not as toxic as produced ethanol.[41,49,53,65,70] For example, a concentration of only 0.5% (w/v) produced ethanol can reduce the growth rate of *S. cerevisiae* ATCC 4126 by 50%.[71] Ethanol produced by *S. cerevisiae* UG5 was found to be 20 to 25 times more inhibitory to its growth rate than was exogenously added ethanol.[53]

Invariably, most authors have claimed that the increased toxicity of produced ethanol was a result of it accumulating intracellularly to concentrations much higher than those seen in the external medium. However, very recent demonstrations that the yeast plasma membrane is very permeable to the movement of ethanol, both in and out,[72,73] suggest that produced ethanol should not be any more inhibitory than added ethanol. A possible explanation for these alterations in the kinetic pattern when yeast produce their own ethanol is that of an osmotic effect due to high substrate concentration.

Mota et al.[49] demonstrated that the growth kinetics of *S. cerevisiae* UG5, *S. cerevisiae* var. *sake*, *S. bayanus*, and *Kloeckera apiculata*, as affected by ethanol, varied with

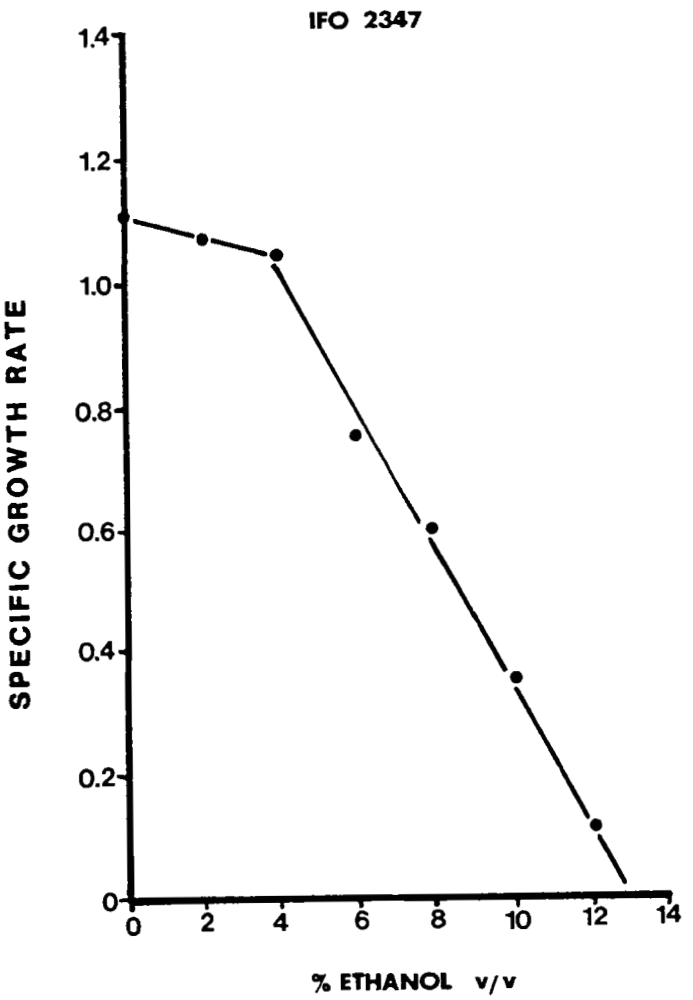

FIGURE 4. Inhibition of growth for *S. sake* IFO 2347 showing decrease in specific growth rate vs. ethanol (v/v). (From Kalmokoff, M. L., Evaluation of Ethanol Tolerance in Selected *Saccharomyces* Strains, M.Sc. thesis, University of Saskatchewan, Saskatoon, 1985. With permission.)

the initial substrate concentration (2 to 25% [w/v] glucose was tested). Inhibition was more pronounced at higher concentrations of glucose. With *S. uvarum* 5D-cyc (and its petite), Brown et al.[74] obtained similar results, finding that inhibition of growth rate by 3 or 6% (v/v) ethanol was more pronounced in a 15% (w/v) glucose medium than in a 2% glucose medium. Increasing inhibition of growth rate by ethanol with higher substrate levels has also been reported by others.[6,41]

It may well be, then, that relative to higher concentrations of added ethanol at low substrate concentrations smaller concentrations of produced ethanol, when combined with the presence of high substrate concentrations, are more toxic. For example, Novak et al.,[53] using *S. cerevisiae* UG5, determined the effects of added ethanol (0.04 to 7.3% [w/v]) on growth rate in a 2% (w/v) glucose medium, but calculated the effect of produced ethanol in 8 to 12.5% (w/v) glucose media. The latter was concluded to be more inhibitory — assuming no substrate effect on the measurements. Such observations, however, may be influenced by a synergistic effect between ethanol and high substrate concentrations on cell growth rate — not a difference in the effect of added or produced alcohol per se. Such a synergistic effect has been reported to inhibit both

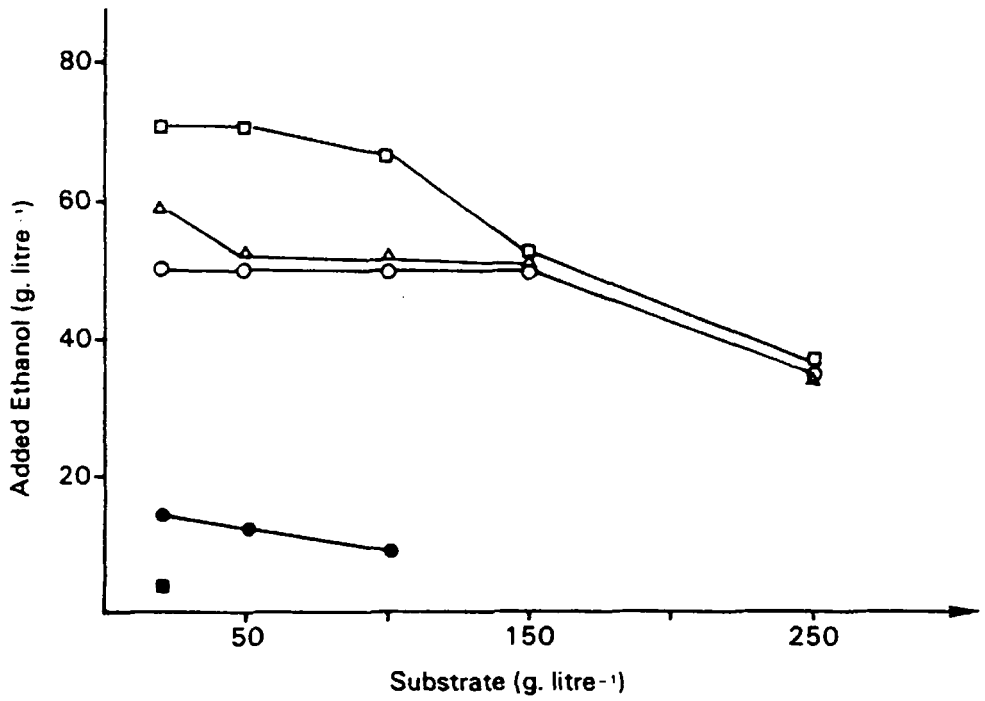

FIGURE 5. Couples of initial sugar and added alcohol which reduce biomass productivities to 50% for the yeasts. ■, *K. apiculata*; ●, *S. uvarum*; ○, *S. cerevisiae*; △, *S. bayanus*; □, *S. cerevisiae sake*. (From Mota, M., Strehaiano, P., and Goma, G., *J. Inst. Brew.*, 90, 360, 1984. With permission.)

viability and fermentative ability.[75] Because researchers generally use lower substrate concentrations when assaying the effect of added ethanol compared to produced ethanol, it is therefore difficult to directly compare the results of one test with another.

Appreciating the fact that ethanol tolerance will vary with initial substrate concentration, Mota et al[49] proposed that rather than measure the effects of added ethanol on growth rate a new parameter should be introduced to quantify ethanol inhibition. They used the factor P/2 — the added ethanol concentration at which biomass productivity (gram per liter per hour) declines to one half of its value relative to that in the absence of ethanol. Using this standard, the authors were able to clearly distinguish varying levels of ethanol tolerance (and how it was influenced by substrate concentration) among the five strains tested (Figure 5). For *S. cerevisiae* var. *sake*, *S. bayanus*, *S. cerevisiae*, *S. uvarum*, and *K. apiculata*, the effects of added ethanol on biomass productivity became particularly pronounced at substrate concentrations exceeding 10 to 15% (w/v).

In addition to substrate effects, growth rate sensitivity to ethanol can also be influenced by the growth phase of the yeast. For example, adding ethanol to wort prior to addition of pitching yeast substantially increased the lag period.[2] Yet similar levels of ethanol, when added to exponentially growing cells, lead only to a reduction in the growth rate, not a cessation of growth.[61,76] It has also been observed that ale yeast viability in a 22°P fermentation only began to decline after the period of logarithmic growth was over and stationary phase was reached.[2] These observations suggest that ethanol is more toxic to stationary phase cells than to exponentially growing cells (although there is some evidence to the contrary).[77,78]

One aspect of ethanol inhibition of growth rate that is not controversial is the widespread agreement that growth rate is considerably more sensitive to the effects of ethanol than fermentation rate.[15,16,54,69,79,80] The most detailed work on this subject has

come from an examination of the effects of added ethanol on *S. cerevisiae* NCYC 479 (a sake yeast) and a lab strain of *S. uvarum*.[16] Ethanol was found to have separate effects on yeast growth, fermentative activity, and viability, with three sets of inhibition kinetics. Cell viability was found to be extremely sensitive to ethanol inhibition, with growth rate, and especially fermentative rate, being more resistant. For both strains, less than 10% inhibition of the growth rate was seen up to 2% (w/v) ethanol (when added to log-phase cells), with complete inhibition occurring at 12% (w/v) ethanol. Fermentative activity, on the other hand, was much more resistant. Kinetics were noncompetitive, but at 12% (w/v) ethanol (where $\mu_{max} = 0$) the fermentation rate was still 25% of the control rate for both strains, and the two strains showed little difference up to 15% (w/v) ethanol. The *S. uvarum* strains showed continued fermentative activity up to 25% (w/v) ethanol, and the sake yeast, up to 30% (w/v).[16]

Similar results demonstrating increased resistance of fermentative activity over growth rate to ethanol have been shown by Pironti[54] (using a rho$^-$ *S. cerevisiae*), Benitez et al.[79] (with numerous wine strains of *Saccharomyces*), and Kalmokoff and Ingledew[15] (studying ale, lager, sake, and bakers' strains of *Saccharomyces* yeast).

3. Ethanol Inhibition of Fermentation Rate

The motivation for research studying the kinetics of ethanol inhibition of fermentative activity has been to produce a model of the overall fermentative process. Mathematical descriptions of this sort are useful for predicting product yields in batch cultures[33,81] and for optimizing productivity in continuous systems.[82,83] As ethanol is known to have a retarding effect on sugar utilization, its inclusion is of obvious importance in any such description.

In 1929, Rahn[55] was the first to propose a model describing the retarding effect of increasing ethanol levels on fermentative activity. He found the rate of decrease in fermentative ability to be directly proportional to the concentration of ethanol. Thus, a straight line relationship was found between the rate of fermentation and the ethanol concentration. Franz[84] found the inhibition to be linearly related to the square of the ethanol concentration. However, his interpretation of the data may not be entirely correct, and it can easily be shown as a direct linear relationship. Linear inhibition kinetics have also been reported by Holzberg et al.,[40] Ghose and Tyagi,[39] Kalmokoff and Ingledew,[15] Lamptey et al.,[45] Casey et al.,[24] and Roman et al.[58]

Holzberg et al.[40] developed kinetic models for the batch fermentation of grape juice using both exponential and stationary phase cells. The rate of change in ethanol production in stationary phase cells was linearly related to the concentration of ethanol up to a level of 10% (w/v). Ghose and Tyagi[65] found that under conditions of rapid fermentation of cellulose hydrolysate (high inoculum, short time) the rate of ethanol production was linearly related to the concentration of ethanol to a level of 11.4% (w/v). Above this value, no fermentation occurred. These results were confirmed in a later study utilizing a chemostat.[39]

The effect of added ethanol on the fermentative ability of a respiratory-deficient bakers' yeast has been evaluated in both batch culture[52] and continuous chemostat.[33] Semilog plots of the rate of ethanol production vs. initial ethanol levels yielded a straight line relationship and led to the development of an exponential function relating productivity and ethanol concentration. However reevaluation of this work in a later paper[32] led to the abandonment of the stated relationship in favor of one in which the inverse of the rate of ethanol productivity was linearly related to the initial ethanol level (hyperbolic). The fact that the data fit both models was thought to be a result of the narrow range of ethanol levels tested (up to 6% [w/v] only).

Casey et al.[24] investigated the effect of beer wort nutrient supplementation on the tolerance of the fermentative ability of a commercial brewing yeast. The reduction in

Table 5
PERCENT ETHANOL (v/v) REPRESENTING THE 50% INHIBITION OF FERMENTATION VALUE (IF_{50})[a] OF WASHED YEAST REMOVED FROM THE ANAEROBIC FERMENTATION OF UNSUPPLEMENTED AND SUPPLEMENTED[b] 27% (w/v) DISSOLVED SOLIDS WORTS

Day	Unsupplemented fermentation	Supplemented fermentation
0	10.1[c]	10.4
1	11.8	11.8
2	11.1	11.5
3	13.2	11.8
4	12.9	11.1
5	11.3	11.1
7	11.9	11.8
9	13.6	N.D.[d]
13	10.1	N.D.

[a] IF_{50} = that concentration of ethanol (v/v) required to inhibit the fermentative power of the control yeast in the absence of ethanol by 50%.
[b] Supplemented with 0.8% (w/v) yeast extract/24 ppm ergosterol/0.24% (v/v) Tween® 80.
[c] Correlation coefficients of the lines relating the % $Q''^{3}_{CO_2}$ value, relative to the control, at the ethanol concentration (v/v) used for the test, were >0.978. Points listed above were taken from these lines as the 50% inhibition point.
[d] N.D. = not determined.

From Casey, G. P., Fermentation of High Gravity Worts by *Saccharomyces uvarum* Brewers' Yeasts, Ph.D. thesis, University of Saskatchewan, Saskatoon, 1984. With permission.

the rate of fermentation was linearly related to the concentration of ethanol and appeared to be independent of the stage of growth or nutritional background of the yeast.[29] Activity was detectable up to a level of 20% (v/v) ethanol, and the point at which a 50% reduction in fermentative activity occurred (IF_{50}, as shown in Table 5) ranged from 10 to 13% (v/v) ethanol.

Kalmokoff and Ingledew[15] increased the scope of these results in a comparison between ale, lager, sake, and bakers' yeasts. For all strains, the inhibition of fermentation rate by ethanol was linearly related to the concentration of added ethanol (Figure 6 illustrates the results for the *S. sake* yeast). When the concentration of ethanol required to reduce the fermentative activity by 50% (IF_{50} value) was calculated, remarkably little difference was seen between the four yeast strains (Table 6), whether assayed at the exponential or stationary phase of growth. Once again, the long-standing belief that sake yeasts are more ethanol tolerant than brewers' yeast did not hold true.

Reports have also appeared on the inhibition of fermentative ability in yeast species other than *Saccharomyces*. Moulin et al.[81] studied the effect of both ethanol (up to 9.6% [w/v]) and substrate on fermentation using a respiratory-deficient and wild-type strain of *Candida pseudotropicalis*. Interestingly, the kinetics of fermentation could be predicted using the model of Aiba et al,[52] and ethanol was found to inhibit fermentative activity in an exponential fashion. Roman et al.[58] found that the rate of ethanol production decreased linearly with increasing the initial ethanol (to a maximum of about 8% [w/v]) in the fermentation of pentose sugars by *Schizosaccharomyces pombe*.

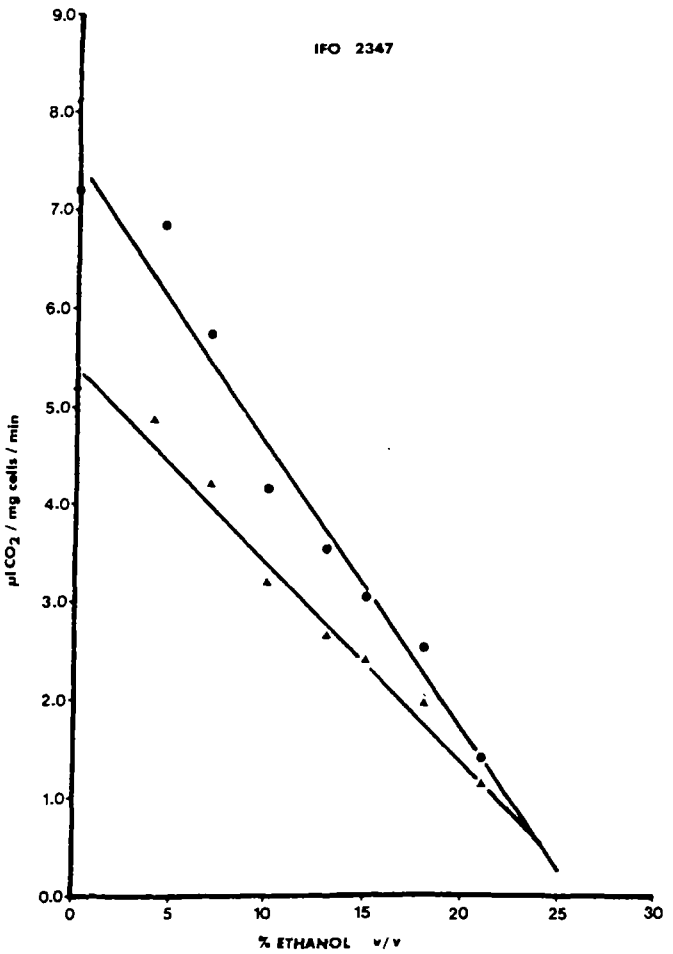

FIGURE 6. Inhibition curve for *S. sake* IFO 2347 showing rate of fermentation (microliters of CO_2 per milligram per minute) vs. percent ethanol (v/v) for both. ●, midexponential and ▲, early stationary phase cell suspensions. (From Kalmokoff, M. L., Evaluation of Ethanol Tolerance in Selected *Saccharomyces* Strains, M.Sc. thesis, University of Saskatchewan, Saskatoon, 1985. With permission.)

4. Overview and Analysis

Since many of the methods used to measure ethanol tolerance are based on assaying ethanol-induced inhibition of the rates of growth and fermentation, it is worthwhile at this point to correlate the above literature with the preceding section.

It is clear that each of the methods used to measure ethanol tolerance differs in terms of the absolute value of ethanol obtained. Growth rate is the first to show sensitivity to ethanol, and growth is completely inhibited by ethanol concentrations of 12 to 13% (v/v). Cell viability exhibits intermediate sensitivity, with significant losses often not occurring until ethanol concentrations that completely suppress growth are reached. Fermentative ability is by far the most resistant to ethanol, and appreciable activity can still be detected even as high as 30% (w/v) ethanol. Clearly, determinations of ethanol tolerance based solely on one method can result in very different conclusions in regards to the tolerance of a given yeast strain. Indeed, some methods now used may significantly underestimate the ability of the strain to produce ethanol.

Table 6
THE LEVEL OF ETHANOL (% [v/v]) REQUIRED TO REDUCE THE FERMENTATIVE ACTIVITY ($\mu\ell$ CO_2/mg/min) BY 50% (IF_{50}) IN BOTH EARLY STATIONARY AND MIDEXPONENTIAL PHASE CELL SUSPENSIONS FOR EACH OF THE TESTED STRAINS

	IF_{50} value	
Strain	Exponential	Stationary
S. sake IFO 2347	13.6 ± 0.5	14.0 ± 0.3
S. cerevisiae NCYC 366	13.4 ± 1.1	13.5 ± 0.3
S. uvarum NCYC 1324	11.4 ± 0.4	13.4 ± 0.2
Bakers' yeast	13.2 ± 0.4	12.9 ± 0.5

Adapted from Kalmokoff, M. and Ingledew, W. M., *J. Am. Soc. Brew. Chem.*, 43(4), 189, 1985.

Additional consideration must also be given to factors which can affect absolute concentrations of ethanol tolerance. Falling under the general heading of cultural and growth conditions, they include such things as medium nutritional composition, starting substrate concentration, temperature, and the use of either added or produced ethanol. Each will influence the final concentration of "tolerance" in the studied yeast, and such tolerance is really only significant or applicable to the exact conditions employed in the assay.

It is the authors' opinion that ethanol tolerance as measured by inhibition of fermentative ability is the best indicator of the potential of a given yeast strain to produce ethanol. This is primarily because the characteristic is not influenced by the nutritional conditions[10,15,24,29] or the growth state of the cells,[15,24,29] and the values correlate well with the upper limits of ethanol production reported industrially and in the literature (i.e., 20 to 23% [v/v] in sake fermentations[25,27]).

B. Ethanol Inhibition of Glycolytic Enzymes

Ethanol is a relatively unique product of metabolism in that it can denature proteins at physiologically produced concentrations.[16] The further observed noncompetitive inhibition of growth rate and fermentation by ethanol leads one to predict that the kinetic pattern for these inhibitions may reflect the effect of ethanol on the specific enzyme(s) of glycolysis having the highest sensitivity towards ethanol.

A commonly suggested key enzyme in this inhibition is hexokinase. For example, Navarro,[85] in a study with *Saccharomyces carlsbergensis,* used each of the glycolytic intermediates from glucose to glyceraldehyde-3-phosphate as the starting substrate. Only when glucose was used was the fermentation rate significantly reduced. This suggested that hexokinase was the most sensitive glycolytic enzyme to ethanol. Others have also demonstrated significant inhibition of hexokinase in yeast by ethanol,[54,86,89] although alcohol dehydrogenase has also been proposed as the glycolytic enzyme most sensitive to ethanol inhibition.[68]

Evidence indicating multiple inhibition sites (as opposed to strictly hexokinase or alcohol dehydrogenase) has been put forward by Pironti[54] and Nagodawithana et al.[87] Pironti[54] found that ethanol inhibited the utilization of both glucose and glucose-6-phosphate in cell-free extracts. Nagodawithana et al.[87] reported noncompetitive inhibition of both hexokinase and alpha-glycerophosphate dehydrogenase, with the overall glycolytic flux controlled by the extent of hexokinase inhibition.

Table 7
DENATURATION OF YEAST GLYCOLYTIC ENZYMES BY ETHANOL — THE % (w/v) ETHANOL REQUIRED TO CAUSE 10, 50, AND 90% DENATURATION IN 30 min UNDER THE CONDITIONS DESCRIBED

	Loss (%)		
Enzyme	10	50	90
Hexokinase	16	19	25
Phosphoglucose isomerase	22	35	>40
Phosphofructokinase	14	19	22
Fructose 1,6-di-P aldolase	15	18	20
Triose phosphate isomerase	25	>35	>40
Glyceraldehyde-P dehydrogenase	13	17	21
Phosphoglycerate kinase	16	19	21
Phosphoglycerate mutase	20	35	>40
Enolase	12	19	28
Pyruvate kinase	18	21	27
Pyruvate decarboxylase	14	17	19
Alcohol dehydrogenase	25	>35	>40

From Millar, D. G., Griffiths-Smith, K., Algar, E., and Scopes, R. K., *Biotechnol. Lett.*, 4, 601, 1982. With permission.

A 1982 study, however, by Millar et al.[90] on the in vitro effects of ethanol on yeast glycolytic enzymes suggested that neither hexokinase nor any other glycolytic enzyme likely played a major role in ethanol inhibition of yeast (Table 7). For example, no inhibition of hexokinase activity was seen below 10% (w/v) ethanol (with inhibition above this level being noncompetitive). Assuming that external concentrations of ethanol during a fermentation reflected internal concentrations, it was concluded that enzyme denaturation was unlikely to play any role at all in ethanol tolerance. For most enzymes, no denaturation was seen up to 15% (w/v) ethanol. Several, i.e., alcohol dehydrogenase, phosphoglycerate mutase, and phosphoglucose isomerase, required the presence of over 40% (w/v) ethanol before complete denaturation was seen. More recently, Larue et al.[91] completely ruled out ethanol inhibition of hexokinase of alcohol dehydrogenase activity as being a significant mechanism of ethanol toxicity in *S. cerevisiae*.

It has been concluded, however, that ethanol inhibition of enzyme activity could play a role in slowing the rate of glycolysis. Inhibition of the activity of the 12 enzymes was not significantly apparent below 5% (w/v) ethanol, but above this, it was competitive for phosphoglycerate kinase, phosphoglycerate mutase, and pyruvate decarboxylase and noncompetitive for the other 9 enzymes. In terms of overall regulation of fermentation rate by ethanol, inhibition of the glycolytic enzymes phosphoglycerate kinase, phosphoglycerate mutase, phosphofructokinase, and pyruvate decarboxylase was likely.

Yeasts with increased levels of glycolytic enzymes (as in the case of respiratory mutants) have been shown by Moulin et al.[51] to have increased resistance to ethanol inhibition of fermentation. Respiratory enzymes have been ruled out as the site of ethanol toxicity affecting yeast growth rate because they are fully functional at ethanol concentrations at which $\mu_{max} = 0$ in *Saccharomyces*.[92] Ethanol can, however, enhance the inhibitory effect of agents known to affect respiratory enzymes.[93]

C. Ethanol Inhibition of Nutrient Uptake

It is widely assumed that the rate of growth in a batch culture is limited to some extent by the rate of entry of solutes into the cell. Ethanol inhibition of growth and fermentation is thought to be related to an interference with this entry process. For example, 0.5 *M* ethanol caused a 74% decrease in the rate of glucose uptake and 55 and 53% inhibition in the rate of glucose uptake of lysine and arginine, respectively, into *S. cerevisiae* NCYC 366. This demonstrated direct inhibition of transport proteins.[61]

Detailed examinations with the yeast *S. cerevisiae* IGC 3507 over the range of 0 to 10% (w/v) ethanol revealed that for the transport of glucose,[47] maltose,[46] ammonium,[94] and amino acids transported by the general amino acid permease[95] ethanol exponentially inhibited the v_{max} (maximum velocity of transport), but had no effect on the K_m (affinity) of the transport proteins for their substrates. The same pattern of inhibition by ethanol was seen for the fructose transport system of *Kluyveromyces fragilis* IGC 2671, but as the inhibition was more severe at lower ethanol concentrations than seen in *S. cerevisiae* IGC 3507, it was concluded to be less ethanol tolerant.[60] Much more, however, will be said on this subject when evidence is presented regarding ethanol effects on yeast membranes.

D. Ethanol Inhibition of Membrane Potential

In 1984, Leao and Van Uden[96] explored the possibility that ethanol and other alkanols (isoproponal, propanol, and butanol) may interfere with membrane potential in *S. cerevisiae* IGC 3507. This work was carried out in order to determine if ethanol inhibition of nutrient uptake in this yeast was in part due to this mechanism of ethanol toxicity. The results showed that the alkanols, with both energized and deenergized cells, did indeed enhance passive proton influx. It was therefore concluded that ethanol inhibition of membrane potential in *Saccharomyces* contributes to the overall inhibition of transport of nutrients whose uptake depends on such a potential. This included such nutrients as maltose, ammonium, and amino acids (e.g., glycine) transported by the general amino acid permease, but not glucose, whose transport is electroneutral.[96]

E. Ethanol-Induced Lipolysis of Cellular Phospholipids

A rather novel proposal of a possible mechanism of ethanol toxicity is that ethanol, at critical concentrations, leads to (or promotes) lipolysis of membrane phospholipids.[97] It was noted that overnight soaking of *S. cerevisiae* cells from an 11°P fermentation in 20% (v/v) ethanol resulted in a 75% decrease in cellular phospholipid content. No effect was seen with ethanol concentrations less than 20% (v/v). If cells were taken from a 20°P wort fermentation and soaked overnight in only 15% (v/v) ethanol, a 50% decrease in phospholipid content was seen. Hydrolysis was suspected, mediated by lipolytic enzymes (present in yeast membrane) which were made more active by ethanol-induced conformational changes in the yeast plasma membrane. Under certain conditions (high gravity brewing, high temperature, high osmotic pressure), it was suggested that intracellular ethanol levels build up to such an extent that phospholipid hydrolysis occurs, causing membrane changes which lead to inhibited cell growth and fermentative activity.[97] As yeasts are now known not to accumulate ethanol to such high levels,[72,73] this mechanism of ethanol toxicity seems unlikely to be significant under industrial fermentation conditions.

IV. YEAST MEMBRANES — THE PRIMARY SITE OF ETHANOL TOXICITY

The plasma membrane is the site controlling the transport of nutrients into the cell

and the excretion of waste products (including ethanol) into the surrounding medium. As early as 1948, ethanol tolerance in *Saccharomyces* was determined to be influenced by its lipid content.[98] As ethanol and plasma membrane lipids are both amphipathic molecules, it is certain that they interact directly with each other during the course of a fermentation, resulting in physiological changes to the membrane. Such changes can influence the tolerance of the yeast towards ethanol, and this section will review the literature on these influences. In particular, emphasis will be placed on the relationship between cellular and medium lipid composition and ethanol tolerance in yeast. Some information dealing with the regulation of yeast growth by oxygen will be presented first in order to facilitate a better understanding of this subject.

A. Regulation of Yeast Growth by Oxygen

Since 1953, it has been known that under anaerobic conditions *Saccharomyces* yeasts require both preformed sterols[99] and unsaturated fatty acids[100] as growth factors. These two compounds are both found in yeast membranes;[101] however, the requirement for these lipids in strains of brewers' yeasts was not found until 1972.[102] The importance of this discovery is that while fermentations are traditionally considered to be anaerobic there must be molecular oxygen (or preformed sterols and unsaturated fatty acids) available to the yeast at some point in the fermentation if it is to proceed normally. Due to the levels of sugars in wort, the role of oxygen as a terminal electron acceptor is at best negligible because of the Crabtree effect.[103] This limits oxygen to the role of a growth factor.

The important anaerobic requirement for oxygen is for reactions involved in the biosynthesis of both sterols and unsaturated fatty acids. The reactions include: the oxidative cyclization of squalene to form lanosterol,[104-106] oxidative demethylation and desaturation reactions in the conversion of lanosterol to ergosterol,[106-108] the synthesis of unsaturated fatty acyl coenzyme-A (CoA) esters from their saturated counterparts,[109,110] and the induction of 3-hydroxy-3-methylglutaryl CoA reductase to convert 3-hydroxy-3-methylglutaryl CoA to mevalonic acid — the first reaction unique to sterol synthesis.[111] Evidence is now available to show that ethanol actually induces the synthesis of cytochrome P-450 in *S. cerevisiae*.[112,113] This may be significant to yeast ethanol tolerance because P-450 is a component of the monoxygenase enzyme involved in the demethylation of lanosterol to ergosterol. This may, therefore, represent an adaptive response of yeast to the presence of ethanol.

Because of this dependency on oxygen for the synthesis of unsaturated lipids under anaerobic conditions, the lipid make-up of yeast membranes will vary considerably with the conditions of yeast propagation and fermentation. Marked differences in the fatty acid compositions of a lager strain of *S. uvarum*, aerated briefly prior to pitching, and the same strain harvested at the end of one fermentation and used directly have been shown.[114,115] The aerated yeast had 90% of its fatty acids as unsaturates (compared to 60% in the nonaerated control yeast), and of these, 41 and 46% were oleic and palmitoleic acid, respectively. Similar results have been obtained with a production ale strain of *S. cerevisiae*.[116]

Sterol concentrations also fluctuate widely in yeast. However, regardless if the growth conditions are aerobic or anaerobic, ergosterol remains the principal sterol in *Saccharomyces* membranes.[101,117-120] Within brewers' yeast strains, the range is normally between 0.1% (anaerobic) and 1% (aerobic) on a dry weight basis.[121] Studies with brewers' strains of *S. uvarum*[122] and *S. cerevisiae*[107,123] indicate that 0.1% is the growth-limiting concentration of sterols in membranes;[107,123] 0.5% is the limiting concentration of unsaturated fatty acids.[122]

While ergosterol is the sterol most often employed to fulfill sterol requirements of *Saccharomyces* yeasts under anaerobic conditions, there is some flexibility in the

choice of sterol. In general, however, the sterol must have a hydroxyl group on C-5,[118] a Δ^{22} double bond,[107,118,124,125] and must contain a 25 β-methyl group.[126,127] Studies with *S. cerevisiae* ATCC 18790[126,127] have shown that at least small amounts of a 24 β-methyl-containing sterol (ergosterol or 22 dehydroergosterol) must be available if growth is to occur under anaerobic conditions (playing a "regulatory" role), but that the "bulk" role of sterols in membranes can be fulfilled with any number of sterols which are C_0-C_2 (α or β) at the C-24 position.[126] It was suggested that reports of successful yeast growth with non-24 β-methyl sterols only[128,129] likely had followed conversion of these sterols using trace amounts of oxygen in the media or cellular C_1 transferase enzymes.[126]

B. Influence of Plasma Membrane Unsaturated Fatty Acid Content on Ethanol Tolerance in Preenriched Cultures

Because of the anaerobically induced requirements in *Saccharomyces* for a sterol[99] and an unsaturated fatty acid,[100] it is possible to produce culture yeasts whose membranes are enriched up to 70% with a specific supplied sterol[124] and up to 55% with a specific supplied fatty acyl residue[17] if a medium with an exogenous source of sterol and unsaturated fatty acid is used.

Based on the assumption that the plasma membrane is the first sensitive organelle to be exposed and interact with ethanol when cells are placed in a solution containing ethanol, a series of studies was carried out with *S. cerevisiae* NCYC 366 to explore the influence of lipid membrane composition on ethanol tolerance. It was shown by Thomas et al.[17] that when membranes were enriched with linoleyl residues ($C_{18:2}$), rather than oleyl residues ($C_{18:1}$) a decline in cell viability when suspended in phosphate buffer with 1 *M* ethanol was always less, regardless of the sterol in the membrane. In addition, this protection by polyunsaturated fatty acid residues was increased even further by incorporating a sterol with an unsaturated side chain (ergosterol or stigmasterol) into the membrane, rather than one with a saturated side chain (cholesterol or campesterol). This is shown in Figure 7. As sterols with unsaturated side chains would have a condensing effect on membrane phospholipids, it was proposed that they increased cell viability by forming a more effective barrier to the external ethanol.

In a subsequent study with the same yeast, Thomas and Rose[61] found that when anaerobically growing log-phase cultures were exposed to 1.5 *M* ethanol the increase in generation time of cultures previously enriched with ergosterol and linoleic acid was less (from 2.5 to 4.8 generations per hour) than with cultures enriched with ergosterol and oleic acid (from 2.4 to 7.7 generations per hour). In the same report, it was also shown that upon exposure to 0.5 *M* ethanol the uptake of labeled glucose, arginine, and lysine was inhibited to a lesser degree in cells with linoleyl- rather the oleyl-enriched membranes.

Clearly then, enrichment with doubly unsaturated fatty acyl residues conferred increased tolerance to ethanol in *S. cerevisiae* NCYC 366, resulting in improved viability, nutrient transport, and excretion of produced ethanol. Since linoleyl-enriched membranes would presumably result in increased membrane fluidity, it somehow compensates better for changes to membrane fluidity induced by ethanol. For example, Thomas et al.[17] proposed that ethanol would decrease membrane fluidity by replacing water molecules around the head groups of phospholipids, thereby decreasing the repulsion between them, with the subsequent decrease in fluidity being better compensated for by $C_{18:2}$ residues rather than $C_{18:1}$. As a universal method of increasing ethanol tolerance in *Saccharomyces*, however, enrichment with linoleyl residues was not suggested. Some of the most ethanol-tolerant strains known (i.e., *S. sake*) were found to contain no polyunsaturated fatty acids in their membranes.[4]

Additional evidence that unsaturated fatty acids play a significant role in ethanol

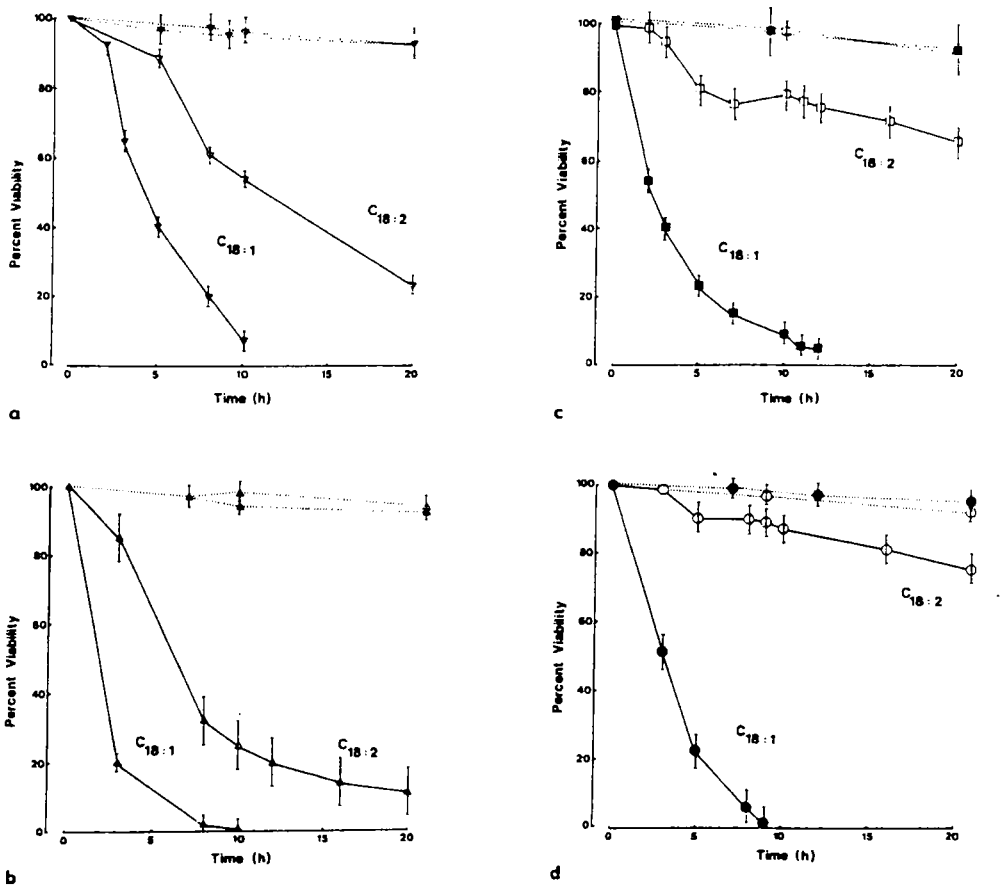

FIGURE 7. Decrease in viability of populations of *S. cerevisiae* NCYC 366 enriched in different sterols and either oleyl (closed symbols) or linoleyl residues (open symbols), when incubated at 30°C in 67 m*M* KH_2PO_4 (pH 4.5) containing (continuous lines) or lacking (dotted lines) 1 *M* ethanol. Viability of populations enriched in campesterol is indicated by inverted triangles (a); cholesterol by upright triangles (b); ergosterol by squares (c); and stigmasterol by circles (d). Vertical bars indicate 95% confidence limits. (From Thomas, D. S., Hossack, J. A., and Rose, A. H., *Arch. Microbiol.*, 117, 239, 1978. With permission.)

tolerance came from a study by Chen[130] comparing the plasma membranes of yeasts normally used in ≈10% ethanol fermentations to membranes in brewers' yeasts. In every case, ethanol-tolerant yeasts from high ethanol fermentations contained a greater percentage of their fatty acids as unsaturated fatty acids, especially with regards to levels of palmitoleic acid ($C_{16:1}$). As a rule of thumb, the stearic ($C_{18:0}$) to oleic ($C_{18:1}$) ratio was always greater than one for brewers' yeasts, but always less than one for ethanol-tolerant yeasts.[130] Such observations may reflect evolutionary adaptation (or selection) by *Saccharomyces* yeast strains subject to continued exposure of high ethanol concentrations.

C. Ethanol-Induced Changes of Plasma Membrane Lipid Composition

Based on studies primarily with *Escherichia coli,* it is quite clear that membrane lipid composition in microorganisms can be significantly influenced by the presence of ethanol. *E. coli* K-12, for instance, as demonstrated by Ingram,[131] contains 26, 37.2, and 34.3% of its fatty acids as $C_{16:0}$, $C_{16:1}$, and $C_{18:1}$ (vaccenic acid), respectively, when grown in the absence of ethanol, compared to 11.5, 33.9, and 50.9% of the same fatty

acids when grown in the presence of 4% (v/v) ethanol. This shift away from saturated to unsaturated fatty acids was demonstrated to be a reversible adaptation in response to ethanol.[131,133] As increased membrane unsaturation would increase membrane fluidity, it was assumed that ethanol must result in decreased membrane fluidity, overcome in this case by adaptive enrichment with vaccenic acid.[131] This viewpoint was reinforced by similar observations in *Tetrahymena pyriformis*,[134] *Mycobacterium smegmatis* ATCC 607,[135] and in the brewers' yeast *S. cerevisiae* NCYC 431,[136] where in all cases ethanol was observed to result in increased membrane unsaturation. In addition, *Zymomonas mobilis*, which can produce over 16% (v/v) ethanol,[137] cannot have its plasma membrane enriched in vaccenic acid as it is already normally over 60% of its fatty acid make-up.[137,138] This suggests a possible evolutionary adaptation to ethanol.

Studies on longer chain alcohols with *E. coli* show that these cause a shift towards saturated fatty acids in the plasma membrane, implying that they decrease membrane fluidity.[139,140] Studies using other biological membranes suggest the same.[141-143] In recent years, however, doubt has been expressed as to the validity of assuming that ethanol decreases membrane fluidity. New evidence suggests that ethanol may in fact increase plasma membrane fluidity. For example, in *Bacillus subtilis*,[144] exposure to ethanol causes an increase in the relative amount of linear fatty acid and a decrease in the relative amount of branched fatty acid — both of which would reduce membrane fluidity. A fluorescent probe study with phospholipid vesicles also reached this conclusion.[145]

To explain this apparent paradox, it was proposed in 1983 that the functionally important part of the adaptative changes to the presence of ethanol was a tendency towards increased chain length (although in favor of unsaturates), which would offset disruptive effects of ethanol.[138] Such shifts towards increased chain length in response to ethanol have been seen in *E. coli*,[131] *M. smegmatis* ATCC 607,[135] and the brewers' yeast *S. cerevisiae* NCYC 431.[136] In addition, extremely ethanol-tolerant strains of *Lactobacillus*, capable of spoiling sake wines containing over 20% (v/v) ethanol, have been found to contain high levels of unusually long chain fatty acids (C_{20}-C_{24}) in their phospholipids.[146,147]

By this theory,[138] the actual net effect of ethanol on plasma membranes is a decrease in membrane integrity (i.e., physical disruption of membrane integrity and structure). Ethanol would weaken the water lattice structure, thereby decreasing the strength of hydrophobic interactions which keep membrane integrity and decreasing the extent of Van der Waals interactions in the membrane interior, thereby increasing the freedom of motion and increasing the polarity of this region. Longer chain fatty acids would increase the surface area for hydrophobic and Van der Waals interactions, restoring plasma membrane integrity in the presence of the ethanol.[138]

In 1984 the debate was resolved by Dombek and Ingram,[148] who demonstrated with cultures of *E. coli* that ethanol actually does cause a net increase in membrane fluidity, largely restricted to near the membrane surface. Longer chain alcohols increase fluidity more deeply in the membrane. Despite enrichment in $C_{18:1}$ vaccenic acid (by growth in the presence of ethanol), isolated plasma membranes had decreased fluidity because of a decrease in the lipid-to-protein ratio in the cells (0.22 vs. 0.51 μmol of lipid phosphorus per milligram protein in cells grown in the presence of 4 and 0% [v/v] ethanol, respectively). Such a change was interpreted to compensate for the combined fluidizing effect of ethanol and the ethanol-induced increase in membrane unsaturation, thereby restoring proper fluidity.[148] Liposomes (containing only lipids) from cells grown in 4% (v/v) ethanol, however, showed increased fluidity as might have been expected due to the increase in $C_{18:1}$ fatty acids at the expense of $C_{16:0}$.[148]

The model put forward was that alcohols act on plasma membranes with their hydroxyl groups located near the surface of the bilayer (H-bonding with polar surface groups, i.e., ester oxygens of lipids, water, or protein). The hydrocarbon chains penetrate towards the center of the bilayer (the longer the alcohol, the further this penetration and the deeper its fluidizing influence), and all alcohols, regardless of chain length, increase fluidity. Ethanol induces a shift towards unsaturated fatty acid synthesis (increasing fluidity), but regulation of the lipid-to-protein ratio in the plasma membrane restores proper fluidity in *E. coli*.[148]

In the same year, results confirming that ethanol increases membrane fluidity in yeast were reported in studies with *Saccharomyces*. Utilizing electron spin resonance studies and *S. cerevisiae* UQM 73Y and PY-1, Curtain et al.[14] compared how ethanol affected the fluidity of protoplasts of the above yeasts (where membranes are intact, containing both lipids and protein) vs. phospholipids vesicles of the same (containing lipids only). Ethanol concentrations of 0.85, 1.75, 2.6, and 3.5 *M* were tested, and both protoplasts and vesicles demonstrated increased fluidity in the presence of ethanol. By utilizing probes with the nitroxide free radical attached to either the 5 or 16 position of the fatty acids (distearoyl phosphatidyl choline [5NL,6NL]), they were able to probe different regions of the membrane. Results confirmed those of Dombek and Ingram[148] that ethanol primarily acts near the membrane surface, with ethanol-induced increases in membrane fluidity being less pronounced further into the membrane.[14] The strains, however, differed in their sensitivity to ethanol, with *S. cerevisiae* PY-1 exhibiting fluidization at ethanol concentrations as low as 0.85 *M*, compared to 2.6 *M* and up in *S. cerevisiae* UQM 73Y.

In apparent contradiction with the model of Dombek and Ingram,[148] it was found that protoplasts (despite containing protein) exhibited enhanced fluidization in the presence of ethanol compared to the vesicles, i.e., the presence of protein did not mitigate the fluidization action of ethanol.[14] This apparent difference between *E. coli* and *S. cerevisiae* has yet to be explained and will remain unclear until attempts are made to see if *S. cerevisiae*, like *E. coli*, adapts to the fluidization effect of ethanol by decreasing its lipid-to-protein ratio.

D. Influence of *Aspergillus* Proteolipid on Ethanol Tolerance

In addition to the influence of membrane composition on ethanol tolerance at the time of exposure to ethanol, there is also considerable evidence that yeast can acquire increased tolerance to ethanol when grown in the presence of certain PLs of *Aspergillus*. The largest contribution of this aspect of ethanol tolerance has resulted from studies on sake yeast, where it has been known for some time that the presence of koji mold mycelia was essential for the formation of high ethanol concentrations — often over 20% (v/v).[13,149-151] Not until the mid-1970s was it demonstrated that the essential component was a PL fraction from the *Aspergillus oryzae* mold.[26,152]

The PL fraction was found to contain phosphatidylcholine as the major phospholipid, linoleic acid as the major fatty acid, and small amounts of sterol, primarily ergosterol.[152,153] The protein moiety was found to serve primarily as a carrier of the lipid constituents, with the high concentrations of ethanol being reached equally well with a phosphatidylcholine-albumin or methylcellulose complex.[26,152,153] When present at 1.5% (w/v) concentration, the koji mold PL complex promoted the production of 20.4% (v/v) ethanol (compared to 17.1% without) by *S. sake* Kyokai No. 7[26] and 18.1% by *S. sake* M-2 (compared to 16.2% without).[154] More recently, *A. awamori* var. *kawachi* mycelia has been used to increase peak ethanol concentration from 18.6 to 20.1% (v/v) by a newly isolated sake strain, *S. sake* w-y-2.[154]

The PL complex has also been found to enhance the "alcohol durability" of yeast, as cultures without it burst during a 48-hr soaking in 20% (v/v) ethanol at 15°C, while

PL-supplemented cells (or cells harvested from an actual sake fermentation) do not.[11] When examined in more detail,[153] it was found that PL "qualitatively strengthened" the membranes, as protoplasts of PL-enriched cells were more stable in 20% (v/v) ethanol than those without (intact PL-supplemented cells did not leak UV-absorbing material over a range of 0 to 20% [v/v] ethanol, while unsupplemented cells leaked even at 10% [v/v] ethanol). The essential component of the PL responsible for alcohol durability was found to be the ergosterol oleate fraction, replaceable by a ternary complex of Tween® 80, ergosterol, and albumin.[155] These were shown to increase peak ethanol production from 17.2 to 19% (v/v) in a 25-day fermentation by *S. sake* Kyokai No. 7.[156]

The use of the PL complex for production of high concentrations of ethanol has not just been limited to sake yeasts. Using the same system of stepwise additions of sucrose to a defined medium with 1.5% (w/v) PL, the ale yeast *S. cerevisiae* IFO 0205 and lager yeast *S. uvarum* IFO 0565 have been shown over a 20- to 30-day fermentation to produce 20.3 and 19.9% (v/v) ethanol, respectively (and 18.3 and 17.7%, respectively, without the PL).[27] Likewise, *S. uvarum* var. *inulyticus*[25] could produce 22.4% (v/v) ethanol in the presence of PL. The PL complex has also been used in corn mash[157] and continuous glucose fermentations[43,158] to increase peak ethanol concentrations and improve yeast ethanol tolerance.

E. Influence of Preformed Sterols/Unsaturated Fatty Acids/Oxygenation on Ethanol Tolerance

In addition to the PL complex of *Aspergillus* molds, other forms of lipid supplements and/or oxygenation have been found to improve yeast ethanol tolerance in numerous alcohol-related industries.

1. Brewery Fermentations

In brewery fermentations, it has recently been demonstrated in the authors' laboratory that the primary factors limiting the production of high levels of ethanol by brewers' yeasts are a combination of nutritional deficiencies in unsaturated lipids and assimilable nitrogen.[23,24,29,30] In North America, the most widely used method to prepare high gravity worts has been the addition of corn syrups to the kettle.[159] Such syrups are virtually devoid of any nitrogenous materials, and their use effectively decreases the proportion of all noncarbohydrate nutrients in the wort.[160] Although worts made only of malt contain excess nitrogen,[161] literature reports with normal gravity worts have illustrated that nitrogen-induced problems, mimicking those found in high gravity brewing, are found when more and more of the extract is substituted with adjunct.[161,162]

Oxygen, as discussed earlier, is required by brewing yeasts for the synthesis of sterols and unsaturated fatty acids.[99,100] Such preformed lipids are present in suboptimal concentrations even in normal gravity worts.[102] In high gravity worts, oxygen availability is diminished even further due to the decreasing solubility of oxygen with increasing wort gravity.[163] As reproductive growth (and rapid fermentation rates) ceases once a limiting value of unsaturated lipids is reached in yeasts,[161] the lowered O_2 solubility in high gravity worts only increases the probability of growth-related attenuation problems. Indeed, in reports where nitrogen and lipid deficiencies were not considered in the fermentation of high gravity worts, problems with protracted and incomplete fermentations have been reported.[2,7,116,164] Incorrectly, the difficulties have been attributed either to ethanol toxicity[7,116] or high osmotic pressure levels,[165] with 16 to 18°P being stated as the limit to high gravity brewing (i.e., 8 to 9% [v/v] ethanol).[159,165-169]

Casey *et al.*,[23] using a commercial lager strain of *S. carlsbergensis*, found that supplementation of 27°P wort with 1% (w/v) yeast extract, 40 ppm ergosterol, and 0.4%

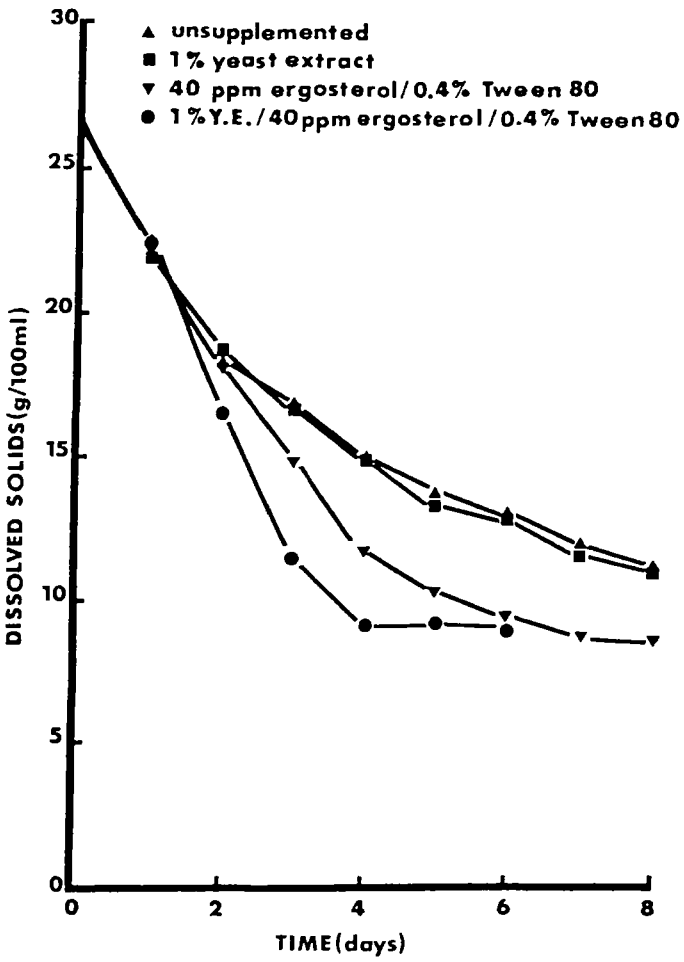

FIGURE 8. Anaerobic fermentation of 27% (w/v) dissolved solids wort with and without nutritional supplementation. A production strain of *S. uvarum* was used. (From Casey, G. P. and Ingledew, W. M., *J. Am. Soc. Brew. Chem.*, 43(2), 75, 1985. With permission.)

(v/v) Tween® 80 (a source of oleic acid) had a dramatic influence on the course of fermentation (Figure 8). Fermentation time was reduced to 4 days (compared to 2 weeks without supplement), and 9.1% (w/v) ethanol was produced. The lipid components of the supplement played a more important role than the yeast extract (although both were required for maximum stimulation). While both factors were required for complete stimulation, the oleic acid alone was much more stimulatory than the ergosterol alone.[29] Casey et al.[24] later reported that the level of supplementation could be reduced to 0.8% (w/v) yeast extract, 24 ppm ergosterol, and 0.24% (v/v) Tween® 80 with no detrimental effect on the degree of stimulation. An elevated pitching rate of 2.2×10^7 CFU/m*l* was used for all worts. This is above traditional rates of 0.5 to 1×10^7 CFU/m*l*, but were used because they had reported early losses in yeast viability and fermentative ability whenever traditional pitching rates were used in very high gravity fermentations.[170] Pitching rates of 1 to 2×10^6 CFU/m*l*/°P of extract have been found to be optimal.[29]

Reduction in the fermentation time was the result of a dramatic increase in the duration and level of cell mass synthesis arising from nutrient supplementation (Table 8). In sharp constrast to the long-held belief in brewing that the bulk of wort attenuation

Table 8
FERMENTATION DATA FROM UNSUPPLEMENTED AND FULLY SUPPLEMENTED 27% (w/v) WORT FERMENTATIONS

	Dry weight (mg/m*l*)		Viability (CFU/m*l* × 10^7)		FAN* utilization (mg/*l*)	
	Unsupplemented	Supplemented	Unsupplemented	Supplemented	Unsupplemented	Supplemented
Start	3.7	3.7	2.2	2.2	213	613
End	9.1	14.0	4.2	6.3	107	344
Change	5.4	10.3	2.0	4.1	106	269

Note: As depicted in Figure 8.

* FAN = free amino nitrogen.

From Casey, G. P. and Ingledew, W. M., *J. Am. Soc. Brew. Chem.*, 2(43), 75, 1985. With permission.

is done by nongrowing cells, it is now clear that the specific rate of sugar utilization by growing yeast cells in a fermentation is substantially higher than that of nongrowing cells.[161] Thus, when the period of new cell mass production ceases during a fermentation, the rate of attentuation also slows dramatically — by as much as 33-fold.[161] It therefore follows that in high gravity brewing, in order to have a rapid fermentation, both the length and level of new cell mass synthesis must be increased over the amounts found in normal gravity brewing.

In the fully supplemented fermentation, yeast cell mass synthesis continued to increase throughout the fermentation, peaking at 14 mg/m*l* (Table 8). In the unsupplemented fermentation, however, the peak value of 9.1 mg/m*l* was reached at day 3, after which the rate of fermentation slowed considerably. Figure 8 suggests that growth-limiting levels of both organic nitrogen and unsaturated lipids were the cause of the protracted fermentation seen in the unsupplemented wort. Levels of free amino nitrogen (FAN) were clearly growth limiting (Table 8), with 26% more FAN being utilized for growth in the fully supplemented fermentation than was even available in total in the unsupplemented wort.

It is significant to note that although the peak viability level was considerably higher in the fully supplemented fermentation no late decline in yeast viability was noted in either fermentation. In fact, relative to peak viability, end viability in the unsupplemented fermentation was still 98.9%, and it was, therefore, not a factor in prolongation of the fermentation time; nor did supplementation increase the fermentative tolerance of this yeast to ethanol (Table 5) as IF_{50} values were virtually identical throughout the fermentations for washed yeast samples from both unsupplemented and fully supplemented fermentations (averaging 11.8 and 11.4% [v/v] ethanol, respectively).[24]

Therefore, supplementation only replenished several growth-limiting nutrients, permitting increased yeast growth which then resulted in the dramatically improved fermentation time. Brewers contemplating the fermentation of very high gravity worts should therefore avoid the practice of merely adding carbohydrate syrups to the existing mash bill as is usually done up to 16 to 18°P,[159] but ideally should include yeast foods as sources of nitrogen and increased levels of O_2 — to allow synthesis of unsaturated lipids. If desired, yeast extract can be replaced in brewing by increased malt content[24] or by other forms of utilizable nitrogen, i.e., ammonium ion or casamino acids.[29] The unsaturated lipids used in this research can be replaced by oxygen sparging

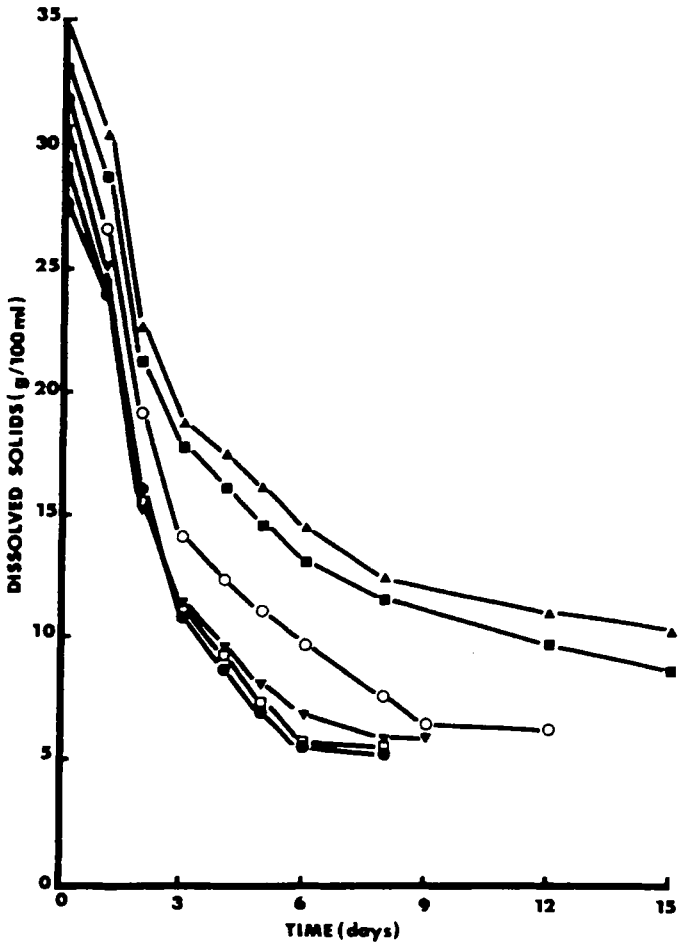

FIGURE 9. Dissolved solids vs. time during the anaerobic fermentation of fully supplemented 27.9 (●), 29.2 (□), 30.9 (▼), 31.9 (○), 33.3 (■), and 35% (▲) dissolved solids wort. (From Casey, G. P., Magnus, C. A., and Ingledew, W. M., *Appl. Environ. Microbiol.*, 48, 639, 1984. With permission.)

or headspace flushing.[24,29] The amount needed is small since only a requirement for membrane constituents exists, not the amount needed in aerobic respiration (cell propagation).

Utilizing this form of supplementation, Casey et al.[24] demonstrated that it was possible to end-ferment maltose adjunct worts up to 32°P, producing 16.2% (v/v) ethanol (Figure 9). Even at such high levels of ethanol, this lager yeast was capable of being repitched over five generations in 28°P maltose adjunct worts (Figure 10), producing an average 14% (v/v) ethanol.[24] The contention, then, that ethanol concentrations of 8 to 9% (v/v) result in yeast crops with such low levels of viability that they cannot be used for repitching[4] is inaccurate. These results also disagree with the commonly held belief in industry that strains of *Saccharomyces* used in brewing have only moderate ethanol tolerance[2] compared to strains used in distilleries.[3] Strangely, even in distilleries, 10% (w/v) ethanol is considered high.

In addition, it was shown that the reason for the brewers' self-imposed limit of 16 to 18°P for high gravity brewing should not be blamed on the intolerance of yeast to ethanol. It would appear that brewers and alcohol manufacturers, using supplementa-

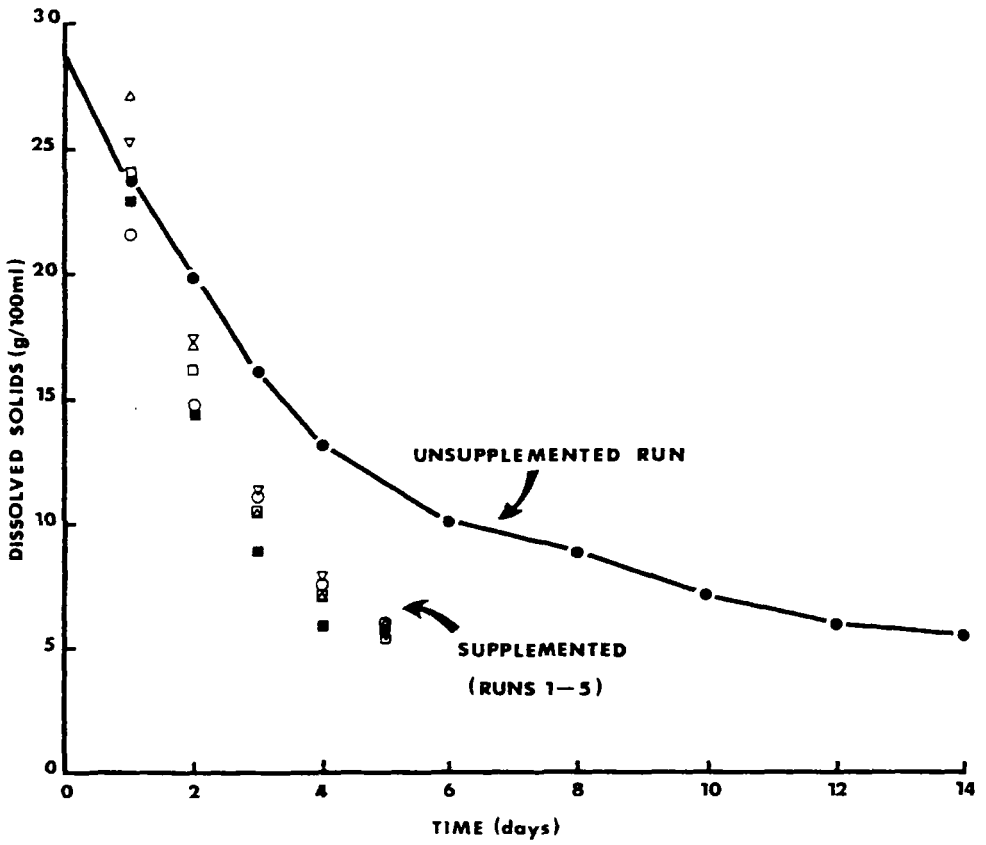

FIGURE 10. Anaerobic fermentation of 0.8% (w/v) yeast extract/24 ppm ergosterol/0.24% Tween 80® supplemented, 28% (w/v) dissolved solids worts over five generations of repitching. □ = generation 1, ▼ = generation 2, △ = generation 3, ○ = generation 4, ■ = generation 5. A single unsupplemented run (●) is included for comparison. (From Casey, G. P. and Ingledew, W. M., *J. Am. Soc. Brew. Chem.*, 43(2), 75, 1985. With permission.)

tion, could easily consider production of "worts" with higher gravities. They would then enjoy larger economies of labor, capital, and energy. The advantages of supplementation to distillers, however, may not reside so much in increased ethanol concentration (since cost savings in distillation would be small), but rather in the rapidity of fermentation, the above-mentioned labor and energy savings, and the use of smaller volumes of water.

2. Vinifications

Results consistent with those found in the fermentation of high gravity brewers' worts have also been reported in vinifications. Although wine yeasts have long been associated with the production of higher concentrations of ethanol than brewers' yeasts, the times required for these fermentations are often very lengthy (up to 3 months in white wine production and 1 month in red wine production). Recently, Ingledew and Kunkee[171] reported that fermentation of grape juice to ethanol levels of 13% (v/v) could be carried out in less than 1 week, even at 14°C, if juice was supplemented with yeast extract and unsaturated lipids (or air) (Figure 11). Thus, the long fermentation times traditionally required in vinifications were not due to the presence of high ethanol concentrations, but rather to nitrogenous and unsaturated lipid deficiencies. If provided for, the extent of yeast growth was increased 30-fold — thereby

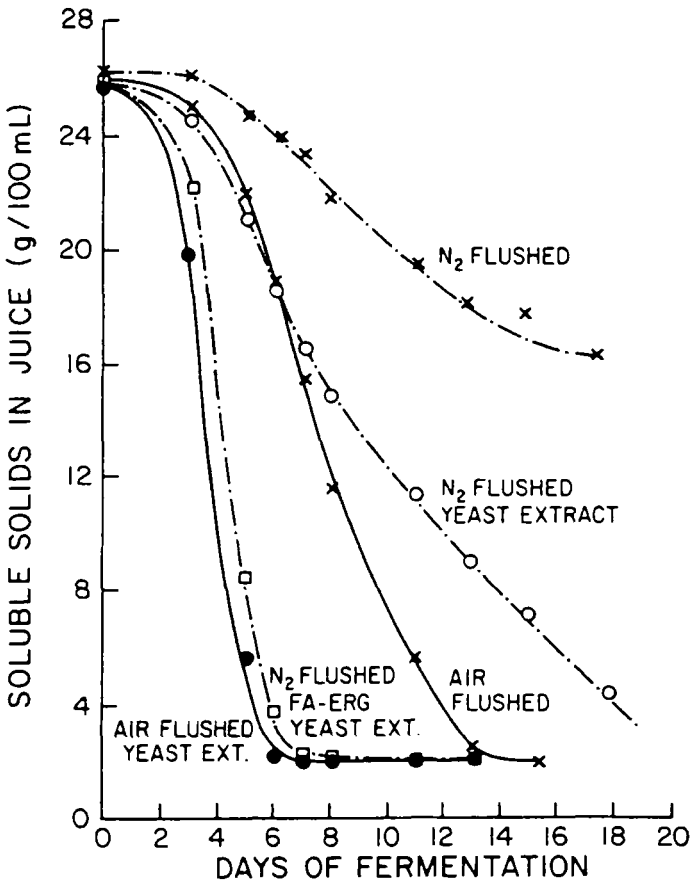

FIGURE 11. Fermentation of supplemented and unsupplemented Ruby Cabernet grape juices under conditions of nitrogen gas flushing of the headspace. (From Ingledew, W. M. and Kunkee, R. E., *Am. J. Enol. Vitic.*, 36, 56, 1985. With permission.)

enhancing fermentation rates and drastically reducing the time required for fermentation.[171] The results are especially pertinent to California Chardonnay grapes, where sluggish or stuck fermentations are not uncommon.[172] Ingledew and Kunkee also predicted that their results would be particularly beneficial to the white wine industry, as processing steps there deliberately exclude unsaturated lipids from the musts (by removing the lipid-rich grape skins) — thereby making the nutrition of white musts poorer than red musts.[173]

It has also been shown in wine fermentations that sterols present initially in the grape must, or added later, can act as "survival factors" for inocula prepared anaerobically. They increase the tolerance of the yeast to ethanol.[174-181] End viability is increased and fermentative activity is prolonged. For example, in 26% (w/v) grape must fermentations fermented at 25°C by *S. cerevisiae*, end ethanol concentrations were 8.3 and 11% (w/v) in unsupplemented and 50 mg/*l* in ergosterol-supplemented fermentations, respectively.[180]

3. Ethanol Fermentations

In batch and in continuous fermentations, the addition of nitrogen and/or oxygen compounds rich in unsaturated lipids or air has also been shown to stimulate fermentation rates and to increase ethanol tolerance.[42,182] Damiano and Wang,[182] using *S.*

cerevisiae, found that adding 2% (w/v) soy flour (rich in protein and lipids) to a 12% (w/v) batch glucose fermentation resulted in a 44% increase in fermentative productivity. The improvement was a result of increased yeast cell growth over the control, and similar results were obtained in continuous culture fermentations.[182] Hoppe and Hansford[42] found in the continuous fermentation of 10% (w/v) glucose that limited aeration (to 0.5% O_2 saturation) increased the ethanol tolerance of the yeast nearly threefold (as measured by the growth inhibition by ethanol).

There is also a claim in the literature by Watson[183] that only a source of unsaturated fatty acid is required to maintain high yeast crop viability in high concentrations of ethanol. An examination of two *S. cerevisiae* and two *S. sake* strains in 30 to 35% (w/v) glucose fermentations revealed that only cultures enriched with oleic acid and/or ergosterol could produce 14 to 15% (w/v) ethanol with high yeast viability. Cultures either unsupplemented or supplemented only with ergosterol produced less than 11% (w/v) ethanol (with poor yeast viability). This led to the conclusion that enrichment with oleic acid alone was all that was required to produce high concentrations of ethanol.[183] To date, the validity of this claim has not been tested utilizing industrial worts.

4. Honey Fermentations

In rapid 25°P Brix honey fermentations[184] at 30°C, *S. cerevisiae* viability was only 2% after 3 hr under anaerobic conditions, during which time ethanol levels rose to 10.1% w/v. By raising the oxygen level to 13% of saturation, 13% of the cells survived, but the ethanol content after 3 hr was lower at 9.6% w/v. When the oxygen level was adjusted to 100% saturation, 60% of the cells survived, but the ethanol level even after 5 hr of fermentation was only 5.7% w/v. At 15°C and with oxygen adjusted to 13% saturation, 94% of the cells survived, but it required 6 hr to reach 9.6% ethanol. With oxygen at 100% of saturation, cells actually multiplied at 15°C, but the ethanol level after 6 hr was only 4.02%.

5. Whey Fermentations

In 20% (w/v) lactose whey fermentations by *K. fragilis*, fermentation time was decreased from 90 to 60 hr, with the production of 10.9% (v/v) ethanol, by adding a Tween® 80 (5 g/*l*)/ergosterol (0.03 g/*l*)/linoleic acid (0.045 g/*l*) supplement.[185]

6. Molasses Fermentations

In the fermentation of 20° Brix molasses solutions by *S. cerevisiae* NSI 113, supplementation with 0.5% (w/v) linseed/cotton seed or soybean oil (or the fatty acids extracted from them) increased peak levels of ethanol (5.9 vs. 4.9% ([w/v]) as well as fermentation rates.[186] In the continuous fermentation of 20% glucose for ethanol production, oxygenation of the yeast strain used was found to nearly double the maximum concentration of ethanol in the effluent — leading to the suggestion that it served to increase ethanol tolerance.[187]

F. Ethanol-Induced Alterations to Membrane Permeability

The presence of ethanol has recently been demonstrated by Ito and Ito[188] to alter membrane permeability in such a way that compounds normally not able to penetrate it do so. The evidence was provided with a *S. cerevisiae* D7M-1 culture where adding 15% (v/v) ethanol to an aqueous suspension of these cells greatly enhanced porphyrin photosensitization. In addition, while porphyrins normally do not penetrate into the cytoplasm of *S. cerevisiae* cells, they do so in the presence of ethanol. Under these conditions, they act as photosensitivity agents as well as mutagenic agents.

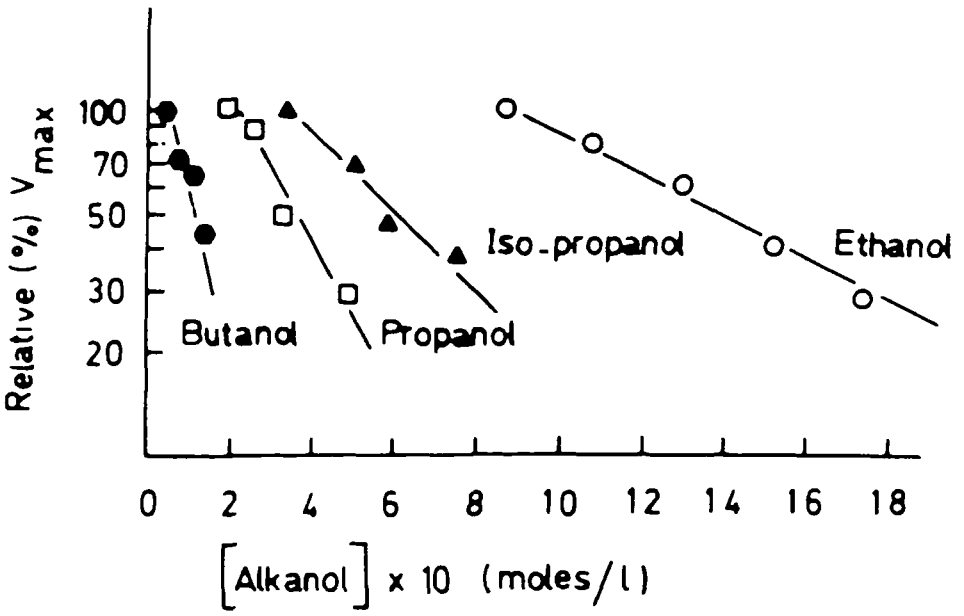

FIGURE 12. Semilog plot of the relative (%) maximum initial uptake rates of methylammonium by a strain of *S. cerevisiae* as a function of alkanol concentration. (From Van Uden, N., Leao, C., Sa-Correia, I., and Loureiro, V., in *Proc. 19th Cong. Eur. Brew. Conv.*, IRL Press, Oxford, 1983, 137. With permission.)

G. Alkanol Studies

Evidence for the involvement of membranes in ethanol tolerance also comes from studies exploring the effects of alkanols of various chain lengths on yeast physiology. It has been shown in transport studies with *S. cerevisiae* IGC 3507 that alkanols (ethanol, isopropanol, propanol, and butanol) act in a noncompetitive manner and exponentially decrease the V_{max} of the transport systems for glucose,[47] maltose,[46] ammonium,[94] and glycine, alanine, tryptophan, tyrosine, and phenylalanine.[95] Figure 12 illustrates this pattern of results for ammonium uptake.[94,229] Similar results have also been reported for the fructose transport system of *K. fragilis* IGC 2671.[60] In all cases, it was found that the more lipid soluble the alkanol, the greater the inhibitory action (Table 9). The concentration of alkanol needed to decrease the V_{max} by one half decreased with the increase in the length and membrane-to-buffer partition coefficient value of the alkanol. This led to the conclusion that the inhibition of these transport systems resulted from nonspecific alkanol-induced changes in the lipid environment of the plasma membrane, suggesting it to be a likely target site of the ethanol toxicity. This conclusion has recently been enhanced by the 1984 results of Leao and Van Uden,[96] showing that the greater the lipid solubility of an alkanol, the more pronounced it is in enhancing passive proton influx across the plasma membrane.

V. ENVIRONMENTAL INFLUENCES ON ETHANOL TOLERANCE

A. Influence of Osmotic Pressure

High substrate concentrations can inhibit yeast growth and fermentative activity directly as a result of high osmotic pressure and low water activity,[189] as well as indirectly as a result of the high levels of ethanol produced during such fermentations. In general, direct substrate inhibition of fermentative ability generally becomes significant somewhere between the range of 15 to 25% (w/v) sugar.[190-192] Complete substrate inhibition

Table 9
ETHANOL INHIBITION OF THE TRANSPORT SYSTEM OF *S. CEREVISIAE* IGC 3507 FOR GLUCOSE, MALTOSE, AMMONIUM, AND AMINO ACIDS TRANSPORTED BY GENERAL AMINO ACID PERMEASE[95,229]

Nutrient	Exponential inhibition constant (l/mol)	Minimal inhibitory concentration (mol/l)
Glucose	0.62	0.33
Maltose	0.65	0.54
Ammonium	1.48	0.87
Glycine	1.69	0.71
Alanine	2.11	0.58
Phenylalanine	2.16	0.53
Tyrosine	2.04	0.66
Tryptophane	1.76	0.56

of fermentation has been reported at 40% (w/v) glucose in batch fermentations with a bakers' yeast and with *S. cerevisiae* ATCC 7754.[193] The nature of the sugar is also important, as substrate inhibition of fermentation by equal concentrations of glucose (180 mol wt) is greater than that by sucrose (342 mol wt).[194] The osmotic pressure exerted by glucose is 1.73 times greater.[194] Substrate inhibition of growth rate, however, usually begins at much lower concentrations than for inhibition of fermentative activity,[195] often beginning at glucose concentrations as low as 5% (w/v).[43]

In brewing, 16°P has been stated as being the upper limit for high gravity brewing, as the osmotic pressure levels encountered in beers greater than this limit would be so high (over 450 psi) as to physically prevent yeast from budding, thereby causing growth and fermentation to prematurely cease.[165] The reports of Casey et al., however, demonstrating that worts of twice this gravity can be fermented, clearly refute this report.[24]

Until recently, little attention has been paid to examining any direct relationship between osmotic pressure and ethanol tolerance. Early papers on the subject noted that as *Saccharomyces* yeasts were acclimatized to higher concentrations of glucose (increased osmotolerance), ethanol tolerance levels decreased.[196] Produced levels of ethanol in high substrate fermentations were considerably lower than the levels of added ethanol necessary to prevent fermentation at low substrate levels.[197] Such observations suggest an interaction between osmotic pressure and ethanol tolerance.

A possible explanation for these observations was not put forward until the effect of osmotic pressure on ethanol production and excretion in a lager strain of *S. uvarum* was explored.[75] Fermentations were carried out in a 10% (w/v) sucrose/yeast-nitrogen base (YNB) medium, with osmotic pressure levels being increased by the addition of sorbitol from 0 to 30% (w/v) in 5% increments. Sorbitol acts to increase osmotic pressure because it is a carbohydrate that can be taken up by brewers' yeasts but not metabolized.[198] It was observed that over the 0 to 30% range, final ethanol concentrations dropped from 4.4 to 2.6% (w/v) due to the inhibition of substrate uptake at higher osmotic pressures. Osmotic pressure was claimed by Panchal and Stewart[75] to cause this inhibition by causing large build-ups in the concentration of intracellular ethanol early in the fermentation. For example, in the 30% sorbitol fermentation, virtually 100% of the ethanol produced during the first 24 hr was stated to be located internally, compared to only 12% in the 15% sorbitol fermentation. By the end of the fermentation, regardless of the level of osmotic pressure, over 95% of the ethanol was located externally.[75]

Also associated with the high osmotic pressure levels were temporary losses in the ability of yeast cells to form colonies.[75] These losses in "viability" were partly reversible, especially if the fermentation was conducted in a growth-promoting medium (sucrose-YNB), rather than in citrate-phosphate buffer. Panchal and Stewart[75] concluded that high osmotic pressure levels hinder the diffusion of produced ethanol to the external medium, with subsequent negative effects on cell viability and fermentative activity. Cellular levels of 0.20 to 0.25 × 10^{-6} mg ethanol per viable cell were reported to be the upper limit before yeast viability became affected, and it was suggested that poor yeast viability in high gravity fermentations may not be due to high levels of external ethanol (or a depletion of essential nutrients), but rather to osmotic pressure effects.[75]

As will shortly become apparent, these results must be questioned. Since it is now known that ethanol crosses yeast membranes by passive diffusion,[73] it is difficult to imagine how high substrate concentrations per se could interfere with the process. In addition, Guijarro and Lagunas,[72] using much higher sorbitol concentrations, reported no osmotic pressure-induced inhibition of ethanol transport or accumulation of high levels of intracellular ethanol. As their methodology for determining intracellular ethanol was considerably more accurate (See Section VI), emphasis should be placed on their results.

In further studies on this subject, Panchal et al.[199] compared the use of mannitol (with sorbitol) to increase osmotic pressure. It was found that mannitol further inhibited the fermentative ability of *S. uvarum* and *S. diastaticus*. The opposite was seen by Ziffer[198] comparing the influence of added mannitol or sorbitol (10 to 40% [w/v]) on the fermentation of 8% (w/v) glucose, nonsaccharified corn mashes by *S. cerevisiae* Y-567. While both sugar alcohols decreased fermentation rate and ethanol production (by increasing the osmotic pressure), the sorbitol was found to be more inhibitory. It was suggested that sorbitol increased osmotic pressure, but also competed with glucose for transport into the cell because it was more structurally related to glucose than mannitol.[198]

Ethanol has also been claimed by Nagodawithana and Steinkraus[68] to accumulate to high levels early in the fermentation of 25°P honey solutions by a *S. cerevisiae* brewers' yeast. Levels up to 2 × 10^{11} molecules per cell were reported (estimated by Steinkraus et al.[272] to be 9.08% [w/v] ethanol), along with significant losses in viability. Osmotic pressure was implicated as the cause, as losses in viability could be offset by adding the honey in increments. For example, after 3 hr at 30°C, viability was only 16% when all 25°P was present at time 0, compared to nearly 90% if the sugar was added in 2.5% increments every 20 min. Larger increments lead to a decreased effectiveness in retaining viability.[68]

In brewing, Casey[29] reported that the sequential addition of adjunct could also result in significant improvements, even without nutritional supplementation. Figure 13 follows the fermentation of unsupplemented 12°P worts containing an addition 12°P in corn syrup extract added at time 0, or in increments at days 1 and 2. Even by adding the extract in only three segments, significant improvements in the rate of fermentation were seen with: (1) 3.6°P more extract being fermented by day 3, (2) 1.8°P lower end gravity being reached, and (3) 6.8 vs. 5.4% (w/v) end ethanol concentration. These improvements were the result of increased yeast growth.[29]

The stepwise addition of carbohydrate (glucose or sucrose) is also an absolute necessity in laboratory sake fermentations if high concentrations of ethanol (20 to 23% [v/v]) are to be reached in defined media.[11,13,25,26,152,153,156,201] In sake fermentations, up to 40% (w/v) substrate is eventually used for the production of ethanol, but if all of this carbohydrate was present at time 0, the sake yeast would ferment at an extremely slow rate and leave considerable amounts of fermentable carbohydrate remaining when fermentative activity ceased (and therefore produce much lower levels of

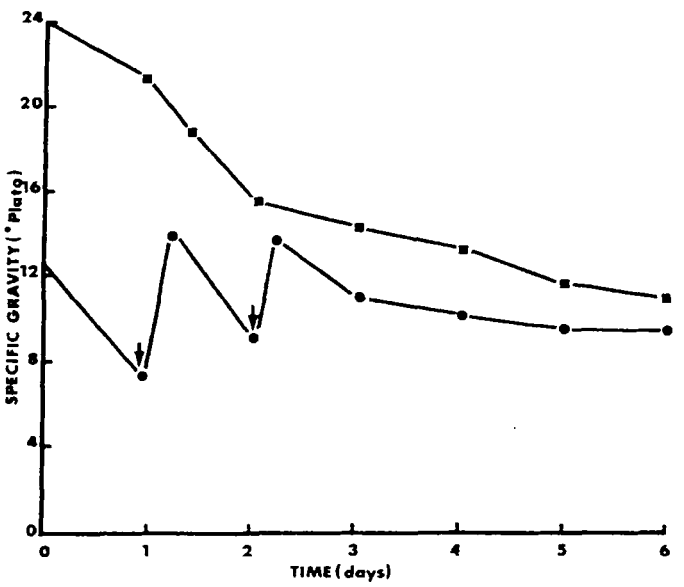

FIGURE 13. Fermentation of unsupplemented 24°P worts. 12°P base wort had an additional 12°P in extract added at time zero (■) or in approximately 6°P increments at days 1 and 2 (●). (From Casey, G. P. and Ingledew, W. M., *J. Am. Soc. Brew. Chem.*, 43(2), 75, 1985. With permission.)

ethanol).[31] In actual sake brewing, the inhibitory effects of high osmotic pressure levels are overcome by successive mashing in three steps, as well as simultaneous saccharification and fermentation of rice carbohydrates.[31]

The sequential or stepwise addition of carbohydrate has also been shown to result in faster and higher levels of ethanol production in other fermentations. For example, *S. cerevisiae* produced 6.6% (w/v) ethanol, with 40% yeast crop viability, in the batch fermentation of 38.6% (w/v) glucose. This compared to 9.5% (w/v) ethanol and 95% end yeast crop viability when 25% (w/v) glucose was present at time 0 and the remaining substrate was infused slowly over an 8-hr period.[202] Similar results were seen in a fermentation with sucrose infusion.[203] Parallel results have been obtained with the bacterium *Z. mobilis* IFO 13756, which produced 12.2% (v/v) ethanol in the batch fermentation of 30% (w/v) glucose compared to 15.8% (v/v) when the same 30% was added stepwise.[137]

In red wine fermentations, levels of 19 to 20% (v/v) ethanol were reported by Cruess et al.[204] when incremental addition of grape syrup was used, compared to a maximum concentration of 16.6% (v/v) ethanol in a straight batch fermentation. This is very close to the ceiling reported by Casey et al.[24] with brewers' yeast in batch fermentation (i.e., 16.2% [v/v]). The smaller the increment, the higher the final concentration of ethanol reached.[205,206] Related to these observations is the Delle equation which states that the sum of the sugar content plus 6 times the ethanol content (w/v) must equal at least 78 for dessert wines to be stable (i.e., 78% [w/v] sugar or 13% [w/v] ethanol).[207]

This synergism between ethanol concentration and osmotic pressure levels in a fermentation (with ethanol being more toxic at high substrate levels) has also been seen in yeasts other than *Saccharomyces*, including *C. pseudotropicalis* No. 513[50] and *C. pseudotropicalis* ATCC 8619.[208] The latter yeast, for example, in a 28% (w/v) solids fermentation (20.1% w/v lactose) gave 12.5% (v/v) ethanol (with nearly all of the lactose

fermented), but only 4% (v/v) ethanol when the whey concentration was increased to 35% (w/v) solids.[208] As the efflux of ethanol from yeast cells is now known not to be hindered by high osmotic pressure levels[72] (with the many reports of high internal levels of ethanol being inaccurate due to errors in methodology), the exact mechanism by which ethanol toxicity is enhanced at high osmotic pressure levels remains a mystery.

An industrial strategy proposed by Pierce et al.[28] and Jones et al.[209] overcomes the increased toxicity of ethanol at high sugar concentrations by fermenting first to 5 to 7% (w/v) ethanol with an osmotolerant yeast, followed by addition of an ethanol tolerant yeast. Such an approach has worked successfully using *S. bisporus* var. *mellis* ATCC 28252 as the osmotolerant yeast and *S. uvarum* ATCC 26602 as the ethanol-tolerant yeast in the fermentation of 29% (w/v) sucrose,[28] as well as in the fermentation of 33% (w/v) sucrose where the two yeasts were co-cultured together, rather than added separately.[209]

B. Influence of Temperature

The optimal temperature for the early stage of a fermentation is usually 5 to 10°C higher than for growth with both temperatures being strain dependent.[210] Mesophilic strains of *Saccharomyces* have optimal temperatures of around 35°C for growth and 40°C for fermentation.[80,211] The presence of ethanol, however, has long been known (in an empirical manner) to alter the relationship that temperature has with growth and the fermentative properties and viability of yeasts.

For example, a reported influence of higher fermentation temperatures is premature cessation of fermentative activity, resulting in incomplete or "stuck" fermentations (fermentable carbohydrate remaining at the end of the fermentation). Concomitantly lower ethanol levels also result.[179,205,206,212] Early studies on fortified wines demonstrated that sequential addition of concentrated grape musts at lower temperatures led to high final ethanol concentrations, e.g., 16.5 and 6.4% (v/v) ethanol at 72 and 99°F, respectively.[205,206] Similar observations have been reported with sake yeasts.[13,25] In addition, lower Delle units were required at higher temperatures to biologically stabilize fortified wines.[192]

Yeast strains used for the commercial production of ethanol also produce lower levels of ethanol at high temperatures. An immobilized bakers' yeast, *S. cerevisiae* ATCC 7754, produced maximum levels of ethanol of 14.5 and 12% (w/v) at 20 and 30°C, respectively, during batch glucose fermentations.[193] *S. cerevisiae* STV 89, in batch 30% (w/v) glucose fermentations, produced 14, 10, and 8% (w/v) ethanol at 20, 35, and 40°C, respectively,[213] and behaved similarly when immobilized.[214] Comparable observations have also been recorded with *S. cerevisiae* NSI 113,[186] *S. cerevisiae* Y-567,[215] and with a thermotolerant strain of *K. marxianus*. In the latter two cases, however, it was reported that the lower peak ethanol values recorded during fermentations at higher temperatures were also in part due to decreased ethanol yields, as well as decreased substrate utilization.

Enhanced toxic effects of ethanol on yeast viability at higher temperatures have also been reported. For example, in the rapid fermentation of honey by *S. cerevisiae*, increased yeast crop viability was seen if the fermentation was conducted at 15°C instead of 30°C.[184] The improvement was said to be a result of decreased accumulation of intracellular ethanol at the lower temperature. At 30°C, internal concentrations of 2×10^{11} ethanol molecules per cell were reported, resulting in logarithmic death throughout the fermentation. This can be compared to levels of 4×10^{10} ethanol molecules per cell at 15°C, a condition under which 100% yeast crop viability was found. Improved yeast crop viability at lower temperatures has also been reported for wine yeasts[192,217] and *S. cerevisiae* STV 89.[213,214] With brewers' yeast, Casey et al.[24] found that as fermentation

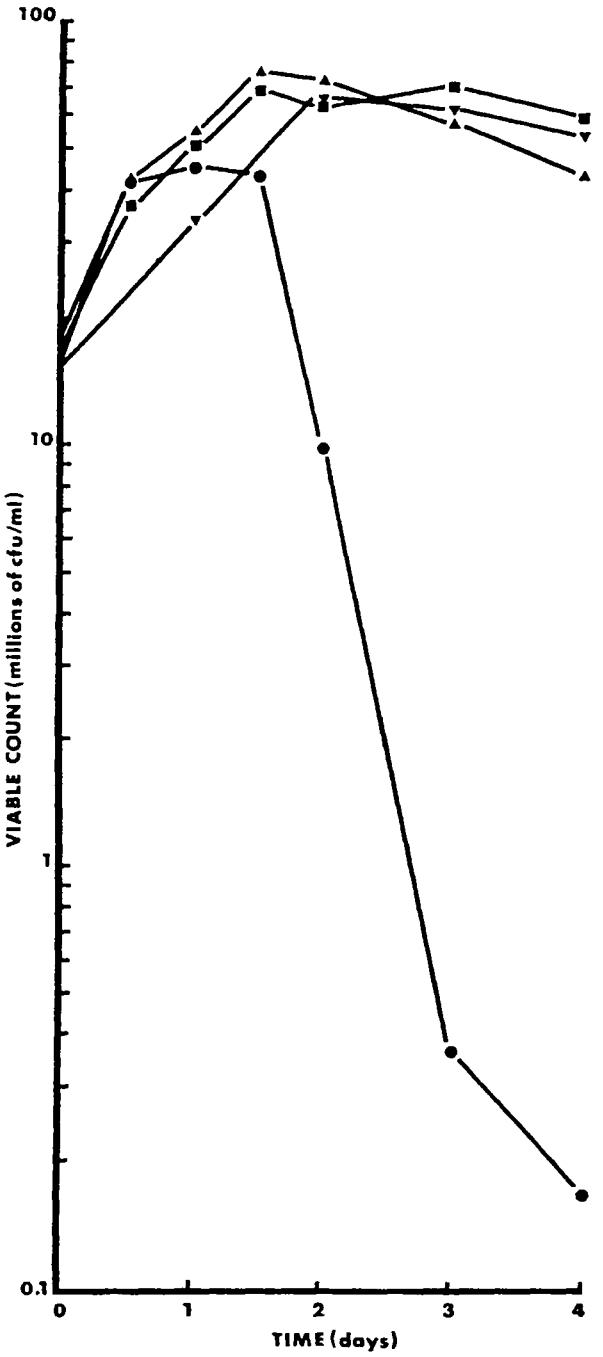

FIGURE 14. Yeast viability levels during the anaerobic fermentation of supplemented 28°P wort at 14 (▼), 20 (■), 25 (▲), and 30°C (●). (From Casey, G. P. and Ingledew, W. M., *J. Am. Soc. Brew. Chem.*, 43(2), 75, 1985. With permission.)

temperatures increased from 14 to 30°C in 28°P fermentations end yeast crop viability declined to a level of 0.1% (Figure 14).

Ethanol effects on yeast growth rate are also known to be more pronounced at higher temperatures. At a fixed concentration of ethanol, the ability of a yeast to resist the inhibitory effect of ethanol on growth rate decreases as temperature increases. Such

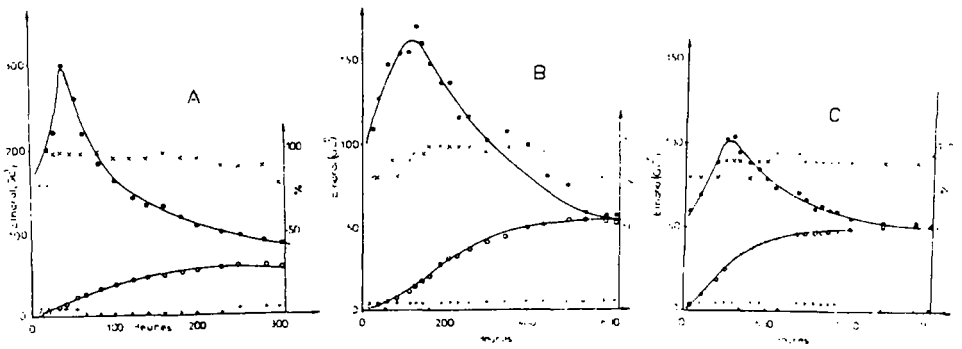

FIGURE 15. Intracellular (●) and medium ethanol (○) concentrations as a function of time at 30 (A), 20 (B), and 10°C (C). (Adapted from Navarro, J. M. and Durand, G., *Ann. Microbiol. (Inst. Pasteur)*, 129B, 215, 1978. With permission.)

observations have been reported with a production strain of *S. sake*,[6] *S. uvarum* ATCC 26602,[80] *S. cerevisiae*,[37,218-221] *K. fragilis*,[221] *K. marxianus* Y KL6 and NCYC 587,[221] *C. pseudotropicalis* YCa9,[221] and *S. uvarum* YSa85.[221] Fermentation rates, however, have been reported to be more resistant to inhibition by ethanol at higher temperatures. For example, over the range of 25 to 45°C, the fermentative activity of *S. cerevisiae* 5D-cyc became more resistant to ethanol inhibition as the temperature increased.[37] Similar results have been reported with *K. marxianus*.[216]

Only recently have attempts been made to determine the mechanism whereby temperature influences the ability of yeast to produce and tolerate ethanol. Initially, as in the case of osmotic pressure, it was claimed that temperature interacted with ethanol tolerance by causing increased accumulations of intracellular ethanol with increasing fermentation temperatures.[69,184] The inhibitory effect of ethanol was therefore claimed to be more pronounced as temperature increased. This phenomenon was most extensively reported by Navarro and Durand[69] in 1978 with a strain of *S. cerevisiae* fermenting 12% (w/v) sucrose at 10, 20, and 30°C. It was claimed that intracellular ethanol concentrations always exceeded extracellular concentrations, with the difference being most pronounced very early in the fermentation (Figure 15). As the temperature increased, peak values of 10, 17, and 30% (w/v) of intracellular ethanol were reached at 10, 20, and 30°C, respectively. Activation energy determinations were measured, and this accumulation was stated to be a result of the resistance of the cell to ethanol diffusion through the membrane to the outside. Significantly, the peak intracellular ethanol values were said to occur just prior to the cessation of growth and prior to the decrease in the specific rate of ethanol production. This led the authors to conclude that these occurrences were due to this accumulation of intracellular ethanol.[69] However, as the methodology employed by these authors to measure intracellular ethanol has recently been shown to result in severe overestimation, this research must be reinterpreted.

A relationship between temperature and ethanol tolerance, and one which provides a realistic mechanism for temperature-related effects on ethanol tolerance, is the effect of ethanol on the minimum, maximum, and optimum temperatures of growth. For example, the wine yeast *S. cerevisiae* Montrachet UCD 522 has its maximum growth temperature at 37°C in the absence of ethanol, but this drops to 32°C in the presence of 6% (v/v) ethanol.[222] Likewise, a survey of 632 strains of wine yeast by Benitez et al.[79] found that while 106 strains could grow in a yeast extract-peptone-dextrose (YPD) medium with 10% (v/v) added ethanol at 22°C, none of them could grow in the presence of 18% (v/v) ethanol at 22°C. This could be lowered to 15% (v/v) ethanol if the temperature were raised to 38°C.[79]

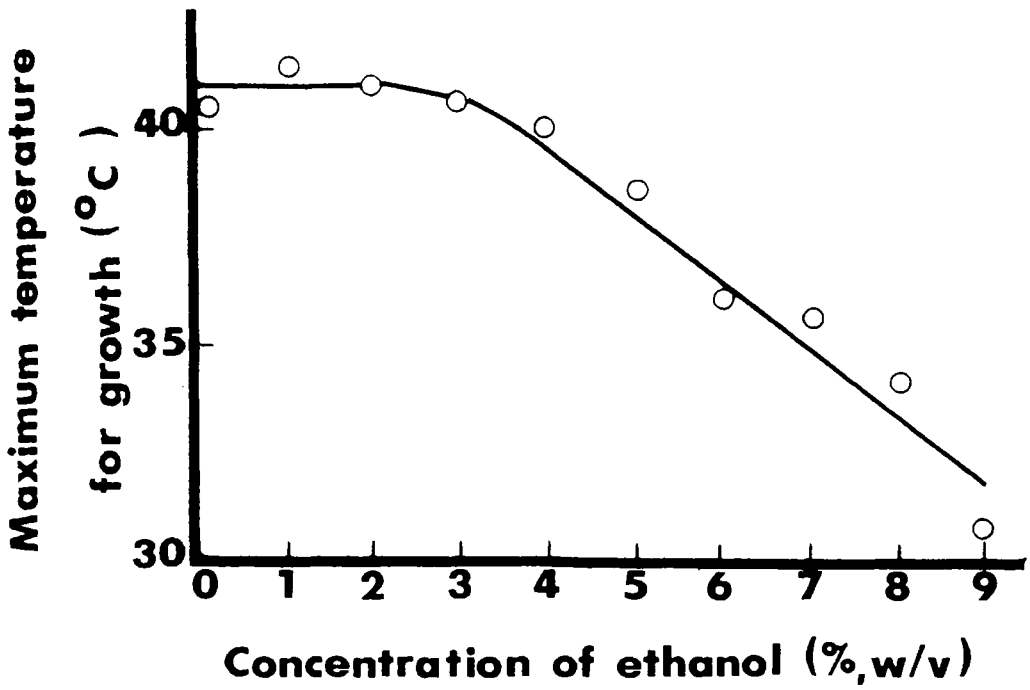

FIGURE 16. Effect of added ethanol on the maximum temperature for growth of a strain of *S. cerevisiae* growing in liquid minimal medium with vitamins and glucose. (From Van Uden, N. and Cruz Duarte, H., *Z. Allg. Mikrobiol.*, 21, 743, 1981. With permission.)

The most detailed studies on this subject, however, have been carried out on *S. cerevisiae* IGC 3507[223-225] and *K. fragilis* IGC 2671.[224,226] The highest ethanol concentrations that still permit growth over a 3 to 45°C temperature range have been examined (for a recent review of the kinetics of thermal death in the presence of ethanol, see Van Uden).[22] Using *S. cerevisiae*, Van Uden and co-workers have found that the maximum temperature of growth began to decrease above 3% (v/v) ethanol (Figure 16).[22,224,225] The minimum temperature for growth began increasing above 2% (v/v) ethanol, and there was a temperature "plateau" of maximum ethanol tolerance between 13 and 27°C at 11% (v/v) ethanol. *K. fragilis* IGC 2671 gave a similar pattern, except that it was less ethanol tolerant, having a similar plateau at only 8% (v/v) ethanol (Figure 17).

The significance of these results to industrial fermentations is readily apparent. At most process temperatures, in the absence of ethanol at the time of inoculation, yeast growth rate will greatly exceed death rate. However, as ethanol accumulates, the optimal and maximum temperatures for growth will decline, moving growth rate and death rate closer to each other. Eventually, an ethanol concentration may be reached at which death rate can exceed growth rate, and fermentation will essentially cease.[22] Whether or not this actually occurs in a fermentation will depend on the process temperature, the ethanol concentrations reached, and the strain involved. The phenomenon is likely to be associated with heat sticking of red wine and fuel alcohol fermentations and could even possibly cause cessation of fermentation in champagne production and high gravity brewery worts if too low a temperature is used (10°C or less).[22,224]

Recent publications have focused on determining which site(s) in the cell, at high temperatures, is made more thermosensitive by ethanol. The general consensus is that the inner mitochondrial membrane is the target.[22,227-229]

Studies by Van Uden[22] with *S. cerevisiae* 1GC 3507 and the alkanols isopropanol,

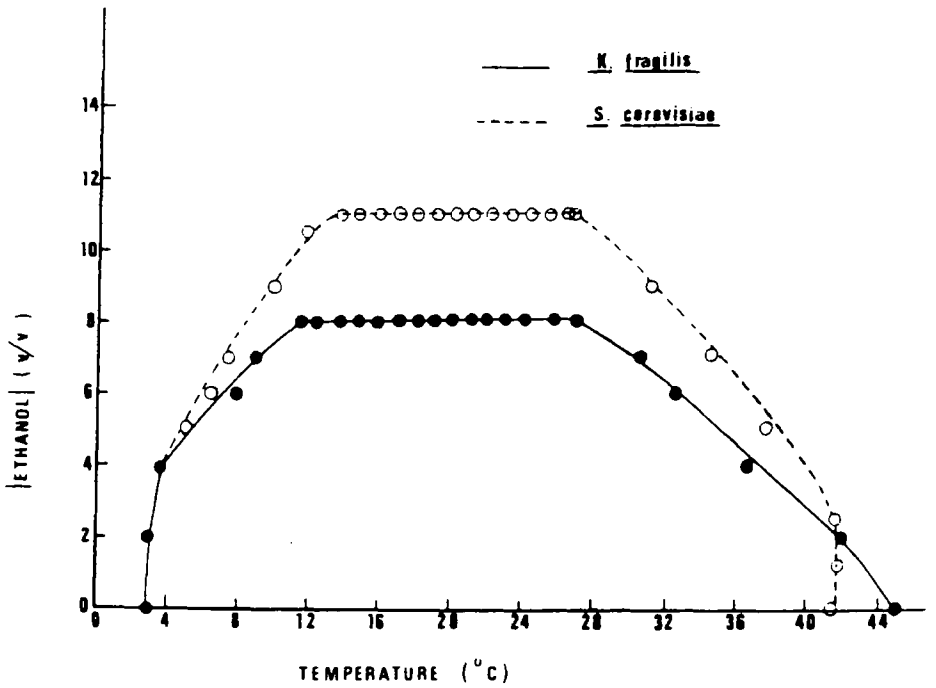

FIGURE 17. Temperature profile of maximum ethanol tolerance of *S. cerevisiae* and *K. fragilis*. Experimental points indicate the concentrations of ethanol above which growth could not be detected.[22,224]

propanol, butanol, and ethanol have shown that the four alkanols tend to make thermal target sites more sensitive. The relevant observation was that the more lipid soluble the alkanol, the lower the concentration required to enhance thermal sensitivity. This suggests that membrane lipids in general are the target sites for alkanol as well as heat sensitivity. The inner mitochondrial membrane in particular has been suggested as the site of toxicity because of the relationship between temperature, ethanol, and the petite mutation in *Saccharomyces* yeast. Exposure of *Saccharomyces* yeast to ethanol results in an increased rate of petite mutation,[227,230] with ethanol shifting both the temperature profile of growth to a lower range as well as that for the petite mutation, without disrupting their relation to one another.[231] Such results suggest that the thermal death sites are identical with or closely related to those governing the maximum temperature of growth (i.e., the inner mitochondrial membrane). Ethanol, therefore, disrupts membranes in such a manner that a smaller quantity of heat results in greater thermal damage to the cell.

VI. ETHANOL TRANSPORT AND THE MEASUREMENT OF INTRACELLULAR ETHANOL IN YEAST

The numerous reports (Table 10) claiming that yeast cells accumulate ethanol internally during a fermentation have only recently added a new dimension to the question of ethanol inhibition and tolerance.[53,61,68,69,75,85,88,136,180,195,203,232-234] This is a matter of considerable controversy because more recent reports on the subject[72,73] completely disagree with the concept of such intracellular ethanol accumulations in yeast. The recent reports are more correct, and the following review of the situation will clearly demonstrate that errors in methodology have been the cause of the controversy.

It has been generally assumed that because of its small molecular size and its solu-

Table 10
LITERATURE REPORTS ON INTRACELLULAR ETHANOL ACCUMULATIONS IN *SACCHAROMYCES* YEAST

Yeast	Medium % (w/v)	Peak intracellular ethanol % (w/v)	Maximum extracellular ethanol % (w/v)	Ref.
S. carlsbergensis	12 (Sucrose)	30	6	69
S. carlsbergensis	12 (Sucrose)	25	6	85
S. cerevisiae	10 (Glucose)	12.7	4.5	53
S. bayanus	5 (Glucose)	13.0	2.5	195
S. cerevisiae	26 (Grape must)	9.4	8.3	180
S. cerevisiae UG5	10 (Glucose)	12.5	4.5	233
S. uvarum	12 (Sucrose)	30.0	6	88
S. cerevisiae NCYC 366	1 (Glucose)	3.0	0.32	61
S. cerevisiae NCYC 431	20 (Glucose)	17	9.5	136
S. cerevisiae UNSW 706800	6 (Glucose)	0.6	0.29	232

bility in membrane lipids ethanol would diffuse very rapidly across biological membranes in response to a concentration gradient.[136,234] In initial publications where internal concentrations of ethanol were measured or approximated, there was, however, universal agreement that ethanol did accumulate during a fermentation and that this accumulation, relative to the external concentration of ethanol, was always greatest very early in a fermentation.[53,61,68,69,75,85,88,136,180,195,232-234]

For example, in the fermentation of 20% (w/v) glucose by *S. cerevisiae* NCYC 431, internal ethanol levels of 4.5 to 5% (w/v) were measured by Rose and Beaven[234] before any ethanol was detected in the medium. Early in the fermentation, when the rates of fermentation were the greatest, the rate of ethanol production exceeded the rate at which it could be excreted to the medium. This accumulation was stated to be due to resistance to its diffusion through the cell membrane.[69] In addition, different yeast strains appeared to have differing abilities to excrete produced ethanol. For example, when the fermentative activity of *S. cerevisiae* UG5 and *S. bayanus* wine yeasts was followed by Strehaiano and Goma[195] in 5% (w/v) glucose fermentations, it was found that both yeasts gave the same yield of ethanol, but the productivity of the *S. bayanus* strain was greater. It was measured as accumulating less intracellular ethanol early in the fermentation (13.2 vs. 10.6%[w/v]) due to a greater ability to excrete produced ethanol. In any case, this apparent early build-up of ethanol was felt by many to contribute to the toxic effects of ethanol on yeast growth and fermentation, a build-up which became aggravated by conditions of high osmotic pressures and temperatures (see below).

There was also general agreement that by the end of a fermentation the difference between internal and external concentrations of ethanol would become considerably smaller. In some cases, the internal concentration was greater than the external concentration, indicating that excretion of ethanol is down a concentration gradient by passive diffusion.[69,88,136,185,195] In others, the internal concentration eventually fell below that of the external medium (up to 18-fold lower[180]), suggesting some form of active transport of ethanol in yeasts.[53,180,232-234]

In the first paper to specifically address this controversy, Loureiro and Ferreira[73]

found that passive diffusion, rather than active transport, was favored for transport of ethanol across yeast plasma membranes. In this paper, within 25 sec of adding ^{14}C ethanol, a steady-state ratio of 0.91 was reached between internal and external ethanol in *S. cerevisiae* IGC 3507. This provided direct evidence that yeast plasma membranes are highly permeable to ethanol and that passage of ethanol through them occurs by passive diffusion in response to a concentration gradient.

It is now clear that the confusion on this subject relates to the methodologies used to measure intracellular ethanol, and only recently has there been critical commentary on the accuracy of these techniques. In general, the methodology used by most researchers was to harvest samples by filtration or centrifugation (possibly including a washing procedure) and to resuspend in a small quantity of the original supernatant (or water) to produce a concentrated cell suspension. Cells were then disrupted mechanically or chemically to release intracellular ethanol, and the ethanol concentration was measured. In some cases,[53,69,85,195] intact cells were injected directly into a gas-liquid chromatograph. The intracellular ethanol concentration was calculated from a knowledge of assumed or calculated cell volumes or water content. If culture supernatant were used to prepare the concentrated suspension, a correction was required for the amount of extracellular ethanol added to the concentrated suspension.

The first criticisms of the washing of cell pellets and the assumption of constant cell volumes came in 1982 by Beaven et al.[136] It was then found that 96.2% of all internal ethanol in a *S. cerevisiae* NCYC 431 sediment was released by a single wash with water, and 93.9% in a single wash with phosphate buffer. The remainder was released within two subsequent washes.[136,234] As washing caused a rapid efflux of ethanol, it was concluded that excretion of ethanol is a downhill process by diffusion in response to a concentration gradient. These results cast doubts on the accuracy of the results of others[62,75] who had used the washing step.

In addition, using tritiated water, which equilibrates with both extracellular and intracellular water, and ^{36}Cl, which equilibrates with interstitial water but does not penetrate the plasma membrane, Beaven et al.[136] found that intracellular water levels do not remain constant over the course of a fermentation (as was assumed prior to 1982). In their case, intracellular water values progressively decreased from 1.93 μl intracellular water per milligram to 1.01 μl/mg dry weight throughout the 64-hr fermentation of 20% (w/v) glucose. The assumptions of 1 g dry weight of cells equaling 3 or 5 ml of intracellular water[53,195,233,234,312] are, therefore, not valid constant relationships, and they would underestimate the amount of internal ethanol, especially late in the fermentation. This would explain the apparent presence of an active transport system for ethanol as reported by some researchers. When data are corrected for these errors, excretion of ethanol is always found to be down a concentration gradient by passive diffusion.[136]

In the latter part of 1983, two additional errors (i.e., the omission of precooling prior to sample centrifugation and the fact that time is required for centrifugation) were pointed out by Dasari et al.[232] Because yeasts continue to metabolize during centrifugation (even at low temperatures) and the metabolites are mostly retained in the sediment, severe overestimates of intracellular ethanol concentrations could therefore result. Any ethanol produced during and after resuspension of the pelleted cells would contribute. It was found during a 30°C fermentation of 6% (w/v) glucose by *S. cerevisiae* UNSW 706800 that if the cells were first precooled with liquid nitrogen for less than 2 min it greatly decreased the estimate of intracellular ethanol compared to cells not precooled prior to centrifugation at 4°C. Dasari et al.[232] also found that increased centrifugation times at 4°C (even with precooling) led to increased estimates of intracellular ethanol. With precooling and short centrifugation times, internal concentrations of ethanol were found to exceed external concentrations by a maximum of only

Table 11
DIFFERENT VOLUMES WHICH COMPOSE PACKED YEAST CELLS

Yeast strain	Growth stage (mg of dry cells/ml culture)	Volume (ml/g of dry yeast cells)[a] Interstitial plus periplasmic space	Volume (ml/g of dry yeast cells)[a] Intramembranous space
ATCC 42407[b]	0.3	1.4	1.7
	1.1	1.3	1.6
	1.5	1.3	0.9
	1.7	1.3	0.7
ACA 174[b]	0.8	1.8	2.0
	9.4	1.8	1.7
	15.2	1.5	1.0
	15.7	1.5	0.9

[a] Samples corresponding to about 25 mg of yeast cells (dry weight) were harvested at the indicated stage of growth and the different volumes were measured.[72] Results are mean values of two experiments.
[b] Yeast cells were grown with 20% (w/v) glucose.

From Guijarro, J. M. and Lagunas, R., *J. Bacteriol.*, 160, 874, 1984. With permission.

one- to twofold (early in the fermentation). These results cast further doubt on papers claiming enormously high levels of intracellular ethanol over 30% (w/v).

It was not until all these criticisms of measuring internal ethanol were incorporated into an experimental design that the controversy was conclusively settled by Guijarro and Lagunas.[72] These authors measured ethanol influx and efflux in *S. cerevisiae* ATCC 42407 and ACA 174. In their protocol, cells were not washed before assaying, but were recovered on membrane filters. Internal ethanol was released by perchloric acid treatment (with >95% recovery being achieved), and intracellular volumes were calculated for every determination. Complete aqueous volumes of packed cells were determined with ^3H_2O, with interstitial and periplasmic cell volumes determined with uniformly labeled glucose. Intramembranous volume was therefore the difference between the two determinations, and the authors were able to show for both strains that as culture age increased, intramembranous space volumes decreased (Table 11). This confirmed earlier observations (Beaven et al.[136]) as well as explained the erroneous reports of active transport systems for ethanol in yeast.

Utilizing [1-^{14}C] ethanol, Guijarro and Lagunas[72] went on to show that ethanol uptake was by passive diffusion and was not carrier mediated because: (1) saturated kinetics for uptake were not observed (first-order kinetics occurred, with an intracellular/extracellular equilibrium being reached in less than 5 sec) (Table 12); (2) ethanol uptake was not inhibited by structural analogs of ethanol (acetaldehyde, propanol, ethylene glycol, and methanol were tested); (3) addition of protein inhibitors (iodoacetate and dimethylsuberimidate) had no effect on uptake; and (4) pHs of 3 to 12 had no effect on ethanol uptake. The above results were also found to be true for ethanol efflux, but of particular importance was the discovery that osmotic pressures up to 66 atm (i.e., 50% [w/v] sorbitol) had no effect on the rapid efflux of ethanol by passive diffusion. In addition, whether in a 2 or 20% (w/v) glucose medium, ethanol accumulation was not found at any stage of growth, with internal concentrations equaling

Table 12
KINETICS OF ETHANOL UPTAKE BY YEAST CELLS

Extracellular [1-^{14}C] ethanol[a] (m*M*)	Uptake time (sec)	Intracellular [1-^{14}C] ethanol[b] (m*M*)
0.0050	0	<0.0002
	5	0.0055
	120	0.0054
0.010	0	<0.0002
	5	0.012
1.0	0	<0.025
	5	1.1
100	0	<2
	5	106
	120	109

[a] *S. cerevisiae* ATCC 42407 cells were harvested at early exponential growth with 2% glucose, washed, suspended in [1-^{14}C] ethanol at the indicated concentration.[72]

[b] After incubation for the indicated times, the radioactivity incorporated by the cells was measured. Concentration of ethanol was calculated with data of Table 11, assuming that this compound was uniformly distributed in the interstitial, periplasmic, and intramembranous space of the cells. If ethanol were exclusively located in the intramembranous space, values two times greater would be obtained.

From Guijarro, J. M. and Lagunas, R., *J. Bacteriol.*, 160, 874, 1984. With permission.

those found externally (Figure 18). In fact, the authors suggested that if the permeability constant of ethanol in plant and animal cell membranes were applicable to yeast cells, the rate of ethanol efflux (3 μmol/g wet weight per second) would actually exceed that of the known rate of ethanol production in *Saccharomyces* (≤ 1 μmol/g wet weight per second). Thus, whether ethanol is produced or added to yeast cultures, there is a rapid establishment of an equilibrium in *Saccharomyces* yeast — independent of osmotic pressure effect (and presumably of temperature as well).

It should be noted, however, that during the early stage of a fermentation, internal levels of ethanol will exceed those found in the external medium. Throughout the course of the ethanol fermentation, the only source of ethanol is intracellularly produced ethanol (estimated by Steinkraus[273] to average 3×10^7 ethanol molecules per cell per second). Therefore, at the start of a fermentation, the level of intracellular ethanol is higher than the extracellular level (which starts at zero). This transitory accumulation of intracellular ethanol continues until external ethanol has accumulated by passive diffusion to internal ethanol levels.

Guijarro and Lagunas[72] convincingly showed that ethanol does not accumulate internally in yeasts. What they did not do, however, was to explain how other phenomena related to ethanol tolerance, and previously implicated with accumulations of intracellular ethanol, actually function. Some reports on osmotic pressure/temperature

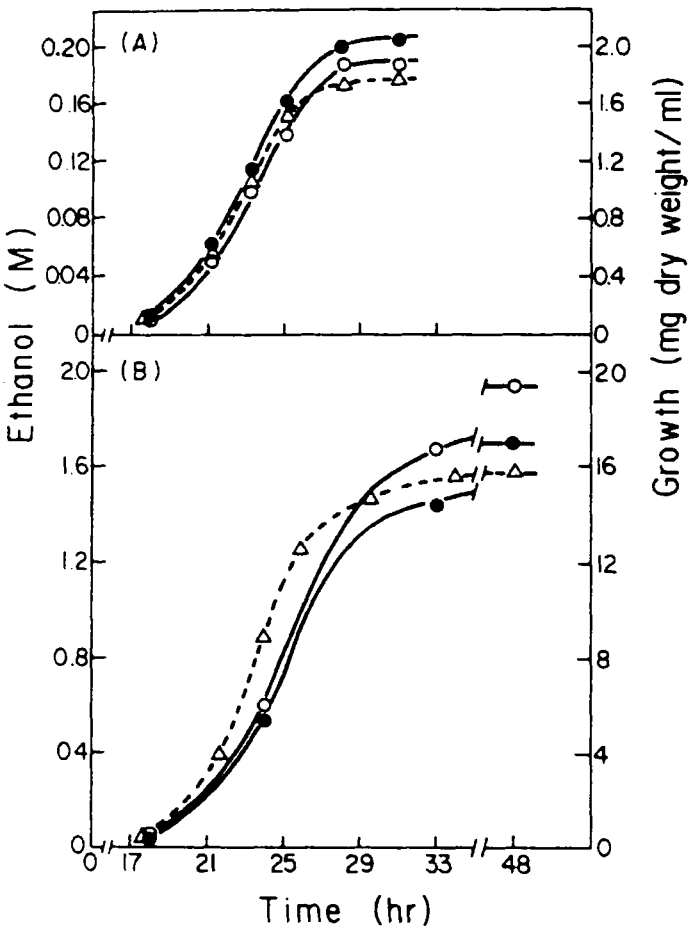

FIGURE 18. Intracellular and extracellular ethanol content at different stages of growth. (A) *S. cerevisiae* ATCC 42407 and (B) *S. cerevisiae* ACA 174 were grown with 2 and 20% glucose, respectively. At the indicated times, samples of the cultures were taken to measure growth (Δ), intracellular ethanol content (●), and ethanol concentration in the medium (O). Ethanol was measured by enzymatic analysis, and its intracellular concentration was calculated with an assumed homogeneous distribution of this compound in the interstitial, periplasmic, and intramembranous space of yeast cells. Mean values of the results of two experiments are shown. (From Guijarro, J. M. and Lagunas, R., *J. Bacteriol.*, 160, 874, 1984. With permission.)

interactions have already been discussed, but there are others as well. For example, Nagodawithana and Steinkraus[68] claim that in 25°P honey fermentations by *S. cerevisiae* yeast ethanol tolerance is greater at lower pitching rates as such yeasts accumulate lower concentrations of ethanol. Other researchers have seen improvements in yeast viability when the pitching rate was decreased during the production of high levels of ethanol.[184,202,235] In addition, certain nutrients have been claimed to lower the accumulation of intracellular ethanol by yeast. For example, Navarro[85] found that the time required to ferment 12% (w/v) sucrose to 5 to 6% (w/v) ethanol by a strain of *S. carlsbergensis* was lowered from 250 to 50 hr by supplementing with either 0.5% (w/v) peptone or 0.5 ppm of a surfactant (a condensation product of myristic acid and lysine). Both were stated to act by decreasing peak internal ethanol concentrations from approximately 20 to approximately 8% (w/v). This resulted in improved and

extended yeast growth (and hence ethanol productivity), resulting in a drastically reduced fermentation time. Similar work was carried out with *S. uvarum*.[88] Vitamin C has also been claimed by Panchal and Stewart[202] to aid in the excretion of endogenously produced ethanol from a *Saccharomyces* brewers' yeast. Thiamin[236] and pantothenate[237] have been reported to improve yeast ethanol tolerance. The exact manner by which any of these nutrients interact with ethanol tolerance remains unexplained.

VII. GENETICS OF ETHANOL TOLERANCE

Surprisingly, little is known about the genetics of ethanol tolerance in yeast. It is clear, however, that under defined conditions different yeast strains differ in their ability to tolerate ethanol[1,2,5,9,15] and that within each strain ethanol tolerance is a reproducible phenomenon.[1,9,15] These properties have been suggested to be of taxonomic value in the classification of *Saccharomyces* yeast.[238]

Considering the complexity of the inhibitory effects of ethanol on yeasts, there can be no doubt that ethanol tolerance in *Saccharomyces* is under polygenic control. Direct evidence for this was provided by Ismail and Ali.[239] They found that segregants derived from diploid strains of *Saccharomyces* exhibited a wide variation in terms of ethanol tolerance, with none of the haploids exceeding the parental level of tolerance. In addition, crosses between different haploids yielded diploids with a wide range of ethanol tolerance, which in some cases exceeded that of the parental strains. Such observations are consistent with polygenic regulation, and because of this it is extremely difficult to isolate ethanol-tolerant mutants by conventional screening and selection techniques.[2,7,9,196] For example, Brown and Oliver[240] have suggested that plating procedures would only be useful for the selection of mutants with increased viability in the presence of ethanol as this is an all-or-none effect. Selection for improvements in fermentative ability and growth would not be possible since these are graded effects.

Ethanol-tolerant mutants of *S. oviformis*, however, have been made by Alikhanyan et al.,[241] who treated cells with the mutagens diethyl sulfate or UV light. Such isolated mutants could grow at ethanol concentrations of 17.5% (v/v), compared to a maximum of 14.4% (v/v) in the wild-type strain. Mutants were also reported by de Mancilha et al.,[269] but no data have been provided for the genetic basis of the increased tolerance.

Interestingly, ethanol itself is a powerful mutagen; concentrations of 24 and 30% (v/v)[230] will induce 10- and 40-fold increases in the rate of petite mutations in *S. cerevisiae*. As the petites in these cases were more ethanol sensitive than the parent strains (based on percent viability after 1 hr in 24% [v/v] ethanol), it was concluded that the increase in the petite mutation rate was a result of mutagenic action, not selection. As petites do not respire, they are, in theory, attractive mutants for the commercial production of ethanol under conditions of low oxygen tension. With petites of *S. uvarum* 5D-cyc[74] and *S. cerevisiae* GRF 18,[242] however, the opposite has been found to be true — both in terms of ethanol yield and productivity. As Brown et al.[74] found, the poorer performance was due to a lower resistance in the petites to the growth inhibitory effect of ethanol (Table 13). It is, therefore, preferable to employ "grandes" for ethanol production, with the provision of only enough oxygen to permit adequate synthesis of unsaturated lipids. It is worth noting, however, that these observations do not necessarily extend to other genera of yeast. For example, Moulin et al.[51] found that *C. pseudotropicalis* petites were more ethanol tolerant than the grandes and were considered more useful for ethanol production under near-anaerobic conditions.

Strains with improved ethanol tolerance have been reported as a result of protoplast fusion experiments. For example, a fusion product of *S. uvarum* and *S. diastaticus* had

Table 13
GROWTH INHIBITION OF *S. UVARUM* 5D-cyc BY ETHANOL

	Grande		Petite	
	Growth rate (μ/hr)	Inhibition %	Growth (μ/hr)	Inhibition %
2% (w/v) glucose				
Control (no ethanol added)	0.312	—	0.254	—
+3% (w/v) ethanol	0.245	21.5	0.156	38.6
+6% (w/v) ethanol	0.123	60.6	0.096	62.2
15% (w/v) glucose				
Control (no ethanol added)	0.285	—	0.22	—
+3% (w/v) ethanol	0.211	26.0	0.116	47.3
+6% (w/v) ethanol	0.074	74.0	0.035	84.1

From Brown, S. W., Sugden, D. A., and Oliver, S. G., *J. Chem. Technol. Biotechnol.*, 34B, 116, 1984. With permission.

improved ethanol-producing capability and the ability to ferment dextrins, as well as increased resistance to the negative effects of high osmotic pressure levels on the rate and extent of fermentations.[199] Likewise, a cross between *S. cerevisiae* TJ1 (a highly flocculent yeast with poor ethanol tolerance) and *S. cerevisiae* N1 (poorly flocculent, but with higher ethanol tolerance) produced a hybrid with good flocculence properties capable of synthesizing 12.4% (w/v) ethanol.[243]

Perhaps the most promising procedure for the selection of a more ethanol-tolerant yeast is that employing continuous culture conditions. In the first report of its kind, Brown and Oliver[240] fermented 15% (w/v) glucose in the presence of 2% (w/v) ethanol. Concentrated ethanol (70% [w/v]) was added to the culture continuously until CO_2 output levels dropped below a certain preset minimum value. Further ethanol addition was not permitted until a preset maximum value of CO_2 output was reached, and, in this way, the culture selected itself. Over the course of the 55-day experiment, it was found that the frequency with which the ethanol pump was turned on increased over the first 30 days, indicating increasing ethanol tolerance with time (Figure 19). Ethanol yields, productivity, and yeast viability also increased during fermentation (Figure 20), and strains could be isolated that were capable of growth in the presence of 12% (w/v) ethanol (unlike the parent strain). Such strains demonstrated higher fermentation rates in the presence of 10, 20, and 35% ethanol (w/v) than did the original yeast (Table 14). Korhola[270] has also selected ethanol-tolerant yeast from continuous cultures. In a related paper, Jones and Greenfield[20] went on to show that short-term adaptive responses to ethanol were also possible. A culture of *S. cerevisiae* UQM 70Y was acclimatized to 7% (w/v) ethanol at 30°C by 16 residence times of growth in a chemostat. Cells were then washed and placed in ethanol concentrations up to 12% (w/v). When viability was followed over a 3-day period, the tolerance of the adapted cells was 40-fold that of the original parent in terms of resistance to cell death. Continuous selection appears to hold great promise for the isolation of highly ethanol-tolerant mutants of industrial *Saccharomyces* yeasts.

In 1983, possible genetic markers for ethanol tolerance in *Saccharomyces* were identified following two types of phenotypic observations. In the first, ethanol tolerance of a wild-type *S. cerevisiae* yeast was compared by Sugden and Oliver[219] to that of the same strain deficient in all three of the major vacuolar proteases (the *pep* 4.3 mutation). At 25°C, 0 to 8% (w/v) ethanol inhibition of the growth rate was virtually identical for the two yeast strains. However, between 30 and 38°C, the same concen-

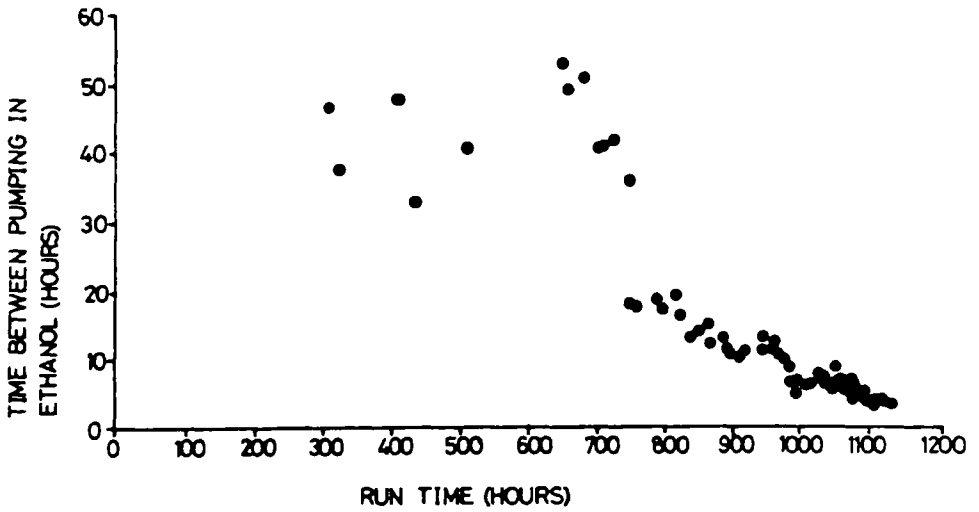

FIGURE 19. Improvement in ethanol tolerance of culture. The graph describes the frequency of switching of the ethanol pump in response to an increase in CO_2 concentration of the exit gas. Operation of the pump was detected by the small downward "blip" on the trace from the CO_2 analyzer which was produced each time ethanol first reached the culture. Each point represents a 7-hr moving average of the interval between operations of this pump. (From Brown, S. W. and Oliver, S. G., *Eur. J. Appl. Microbiol. Biotechnol.*, 16, 119, 1982. With permission.)

trations of ethanol produced significantly greater inhibition of growth in the *pep* 4.3 mutant. It was speculated that the *pep* 4.3 mutation resulted in generalized changes to yeast membranes. Yeast with such altered membranes had decreased ethanol tolerance.

The second report described a relationship between ethanol tolerance and heat shock proteins. Such proteins were initially found by Plesset et al.[244] to be induced by preincubation of the yeast in 1.55 *M* ethanol for 20 min at only 23°C, rather than (as normally required) preincubation at elevated temperatures (36 to 41°C). In studies with *S. cerevisiae* ATCC 26422, a sake yeast, Watson and Cavicchioli[18] went on to show that possession of the ethanol-induced proteins conferred significantly increased ethanol tolerance. Yeast viability was 40% in 24% (w/v) ethanol after 36 hr compared to 0% within 32 hr in the same yeast without the heat shock proteins. Such cultures were also better able to resume growth once the ethanol was removed.[18] Subsequently, it was shown[245] that cultures of *S. cerevisiae* CBS 1171, 1237, 1242, and *S. cerevisiae* ATCC 26422, which had heat shock proteins induced in the absence of ethanol, also had increased resistance to ethanol-induced losses in viability (Figure 21). The possibility therefore seemed to exist that cloning of the genes responsible for heat shock proteins into yeast could be one means to increase ethanol tolerance.

This possibility was later ruled out[21] when the same authors went on to show that neither mitochondrial nor cytoplasmic protein synthesis was required for the heat shock acquistion of increased ethanol tolerance in *S. cerevisiae* ATCC 26422. This phenomenon therefore remains unexplained, but Watson et al.[21] did speculate that perhaps heat shocking places yeast in a temporary "dormant state" that makes them better able to resist ethanol-induced loses in viability.

VIII. INDUSTRIAL STRATEGIES TO MINIMIZE ETHANOL TOXICITY

A. The Vacuferm Process

Vacuferm fermentations involve the continuous fermentation of sugars under vacuum. Ethanol is allowed to boil off as it is made, eliminating the possibility of inhibi-

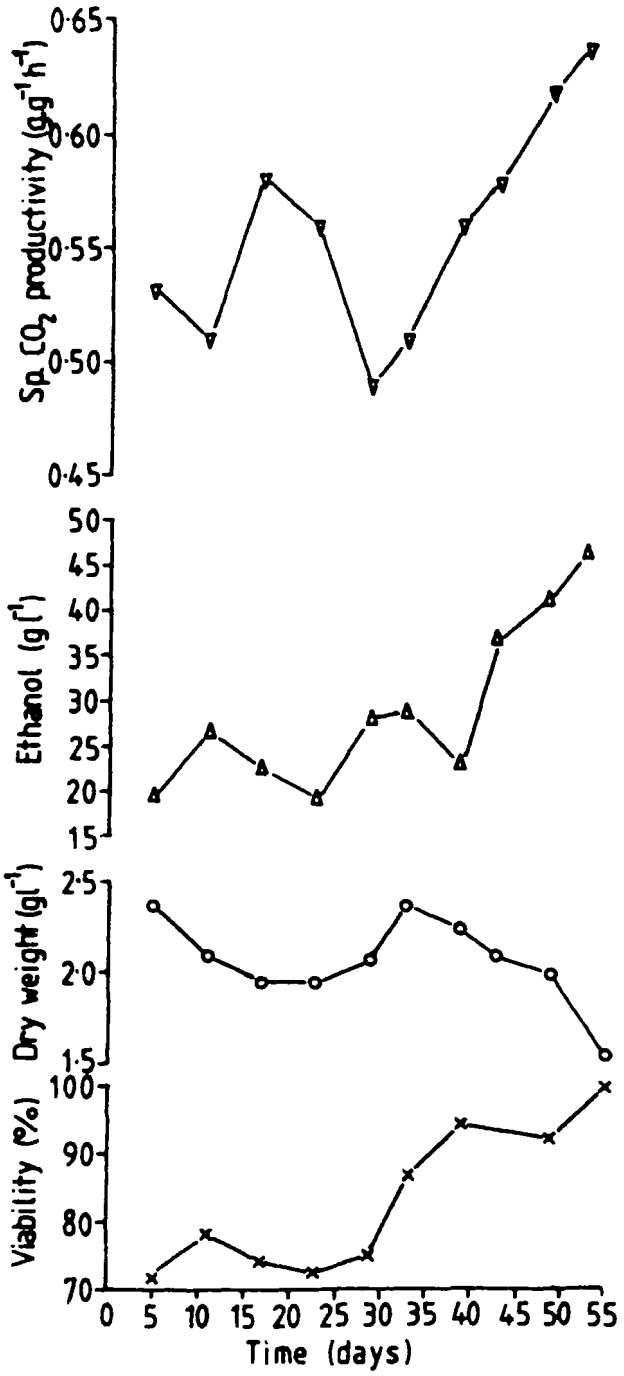

FIGURE 20. Changes in the performance of the culture during the selection experiment. X--X, cell viability; O--O, dry weight; Δ--Δ, ethanol concentration; ∇--∇, specific rate of production of CO_2. These parameters were measured every 2 days, and the graph shows the arithmetic mean values for successive 6-day periods. (From Brown, S. W. and Oliver, S. G., *Eur. J. Appl. Microbiol. Biotechnol.*, 16, 119, 1982. With permission.)

Table 14
THE FERMENTATION PERFORMANCE OF *S. CEREVISIAE* WILD-TYPE (5D-cyc) AND FIVE ETHANOL-TOLERANT MUTANTS WHICH WERE ISOLATED FROM A CONTINUOUS FERMENTOR 1133 hr INTO THE SELECTION EXPERIMENT

Strain	Control $Q^{\nu}_{CO_2}$	Percent of control in presence of ethanol		
		10% (w/v)	20% (w/v)	35% (w/v)
Wild-type	0.822	74.6	71.9	66.0
154	0.823	80.5	71.1	77.7
155	0.793	78.8	70.7	77.7
158	0.993	80.7	76.8	73.8
159	1.33	93.4	80.7	75.8
160	1.74	89.4	83.5	74.6

Note: Specific fermentation rates ($Q^{\nu}_{CO_2}$) of cells withdrawn from exponential phase cultures of these six strains grown in YEC medium were determined. The control value was obtained using 5% (w/v) glucose and no added ethanol. The inhibitory effect of 10, 20, and 35% (w/v) ethanol was determined under the same conditions and is presented as a percentage of the control value for each strain.

From Brown, S. W. and Oliver, S. G., *Eur. J. Appl. Microbiol. Biotechnol.*, 16, 119, 1982. With permission.

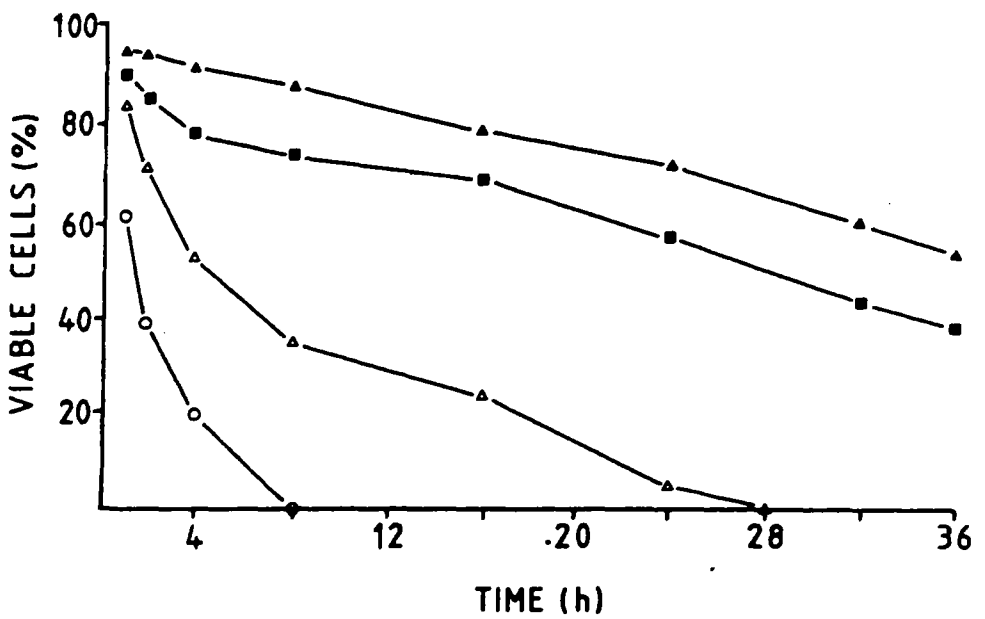

FIGURE 21. Primary heat shocked-induced ethanol tolerance in strain ATCC 26422. The ethanol concentration was 14% (w/v). Δ, control cells, 23°C; ▲, primary heat-shocked cells, 37°C/30 min; O, heat stressed cells, 23° to 52° C/5 min; ■, secondary heat-shocked cells, 37°C/30 min immediately followed by 52°C/5 min. In this and all subsequent experiments, cells were cooled to 23°C before addition of ethanol. (From Watson, K., Cavicchioli, R., and Dunlop, G., in *Proc. 18th Conv. Inst. Brew. (Aust. and N.Z. Sect.)*, Clark, B. J., Harvey, J. V., Itzcovitz, S., and Wheatland, G. W., Eds., Institute of Brewing, Sydney, 1984, 229. With permission.)

tion of yeast growth and fermentation.[246-248] Vacuums of 50 mmHg at 35°C[246,247] and 32 mmHg at 30°C[248] are required. In the former case, productivities of 82 g of ethanol per liter per hour were reported if cell recycling was included (returning yeast harvested from the overflow to the fermentor in order to maintain high cell densities). This compares to productivities of 40, 29, 7, and 2.2 g of ethanol per liter per hour in continuous vacuum fermentation without cell recycling, in continuous fermentation with cell recycling only (no vacuum), in continuous fermentation (no recycling or vacuum), and in traditional batch fermentations, respectively.

In a commercial modification of the Vacuferm system, called the Biostil system, ethanol inhibition is overcome by removing the liquid (the yeast is retained) from the fermentor before inhibitory levels of ethanol are reached, distilling off the ethanol, and returning to the fermentor the liquid for continued fermentation (along with some additional fresh substrate).[249] A similar system, called Flash Ferm,[250] "flashes" off the ethanol from the removed liquid by vacuum, not distillation.

B. Extractive Fermentations

Ethanol inhibition of fermentation can be overcome by the selective extraction of ethanol with nonpolar solvents during a fermentation. For example, using dodecanol extraction, the ethanol-producing productivity of *S. cerevisiae* UG5 was increased fivefold, allowing the use of 40.7% (w/v) glucose feeds, instead of a previous maximum of 20 (w/v).[251,252]

Other solvents, including various ketones, esters, higher alcohols, amines, and chlorinated hydrocarbons, have been suggested.[253-255] Difficulties encountered with the use of extractive solvents include toxicity to the yeast,[254] toxic accumulations of feed salts,[253,256] and toxic accumulations of secondary byproducts of metabolism (including acetaldehyde, glyceraldehyde, formate, lactate, acetate, 1-propanol, and 2,3-butanediol).[257] One means proposed to overcome solvent toxicity is to construct a physical barrier between solvent molecules and immobilized cells. Matsumura and Markel[254] have proposed Poropack Q (100 to 120 mesh) for this purpose as it does not retain ethanol. Indeed, in tests with *sec*-octanol (normally very toxic to yeast), culture viability remained very high. A difficulty with this procedure, however, is that with time, the adsorbent becomes saturated with solvent molecules and the barrier function becomes lost.

C. Selective Adsorption

The selective adsorption of ethanol by solids has also been proposed as a means by which to overcome ethanol toxicity during a fermentation. While proposals are still very preliminary, recommended solids for such a purpose include divinyl benzene, cross-linked polystyrene resins,[258] and IRC-50-activated carbon resins.[257] These solid sorbents have a higher selectivity for ethanol than water. Desorption could later be carried out by stripping the ethanol with a carrier gas, e.g., nitrogen.[258]

D. Use of Specialized Filters

Ethanol toxicity has been reported to be overcome in fermentation vessels by using microporous filters, which retain yeast yet allow the liquid (containing ethanol) to pass through.[259-261] The liquid can then be distilled and returned for continuous fermentation. Polyamides and acrylonite polyester membranes have also been suggested by Toledo[262] for separating ethanol from water because they allow the passage of water, but not ethanol. Difficulties with the technique are that they also result in the accumulation of toxic feed and metabolic byproducts.

E. The Ex-Ferm Process

This process, developed in Guatemala by Rolz and Cabrera,[263] uses pieces of sugar cane as the raw substrate. It involves the use of a mixed solid-liquid phase fermentation, where there is simultaneous extraction and fermentation of sugar cane in a rotating drum fermentor. In this method, osmotic pressure is low and the produced ethanol is therefore not as toxic as the same ethanol would be if all of the cane sugar were present initially. The system has been found to operate at high ethanol productivity levels with various strains of *Saccharomyces*.[263,264]

F. Yeast Immobilization

An unanticipated result of yeast immobilization is that ethanol and substrate tolerances of immobilized yeast are greater than those of their freely suspended counterparts. For example, Holcberg and Margalith[193] found that *S. cerevisiae* ATCC 7754 entrapped in K-carrageenan in batch 30% (w/v) glucose fermentations could produce a maximum ethanol concentration of 12.1% (w/v) (with 56% yeast crop viability) compared to only 9.6% (w/v) (with 28% yeast crop viability), if not immobilized. The same authors showed similar improvements in ethanol production and tolerance using sodium alginate or gum arabacum for entrapment.[66] Similar improvements in ethanol tolerance by immobilization have been reported for a strain of *S. carlsbergensis*,[265] *S. cerevisiae* NRRL Y-132,[45] and *S. diastaticus*.[266]

Suggestions have been made that immobilization improves yeast performance by creating a "protective layer" around the cells in which there is lowered alcoholic stress in the microenvironment (by facilitating more efficient ethanol excretion). Also, a sugar gradient exists, with the concentrations in the yeast microenvironment being lower than those in the external medium.[66] Evidence for this theory, at least with regards to improved ethanol tolerance, comes from the observation that added ethanol is more toxic to immobilized yeast than freely suspended yeast.[66] This suggests that the polymers used for immobilization assist the passage of ethanol both in and out of cells, providing indirect evidence that immobilization allows yeast to more efficiently excrete ethanol.

IX. SUMMARY

It is now certain that the inherent ethanol tolerance of the *Saccharomyces* strain used is not the prime factor regulating the level of ethanol that can be produced in a high sugar brewing, wine, sake, or distillery fermentation. In fact, in terms of the maximum concentration that these yeasts can produce under batch (16 to 17% [v/v]) or fed-batch conditions, there is clearly no difference in ethanol tolerance. This is not to say, however, that under defined conditions there is no difference in ethanol tolerance among different *Saccharomyces* yeasts. This property, although a genetic determinant, is clearly influenced by many factors (carbohydrate level, wort nutrition, temperature, osmotic pressure/water activity, and substrate concentration), and each yeast strain reacts to each factor differently. This will indeed lead to differences in measured tolerance. Thus, it is extremely important that each of these be taken into consideration when determining "tolerance" for a particular set of fermentation conditions.

The manner in which each alcohol-related industry has evolved is now known to have played a major role in determining traditional thinking on ethanol tolerance in *Saccharomyces* yeasts. It is interesting to speculate on how different our thinking on ethanol tolerance would be today if sake fermentations had not evolved with successive mashing and simultaneous saccharification and fermentation of rice carbohydrate, if distillers' worts were clarified prior to fermentation but brewers' wort were not, and if grape skins with their associated unsaturated lipids had not been an integral part of red wine musts.

The time is now ripe for ethanol-related industries to take advantage of these findings to improve the economies of production. In the authors' opinion, breweries could produce higher alcohol beers if oxygenation (leading to unsaturated lipids) and "usable" nitrogen source levels were increased in high gravity worts. White wine fermentations could also, if desired, match the higher ethanol levels in red wines if oxygenation (to provide the unsaturated lipids deleted in part by the removal of the grape skins) were practiced and if care were given to assimilable nitrogen concentrations. This would hold true even at 10 to 14°C, and the more rapid fermentations would maximize utilization of winery tankage. Distilleries and commercial ethanol producers could likely achieve higher ethanol concentrations in their mashes if higher wort sugar levels were produced and if the fermentation temperature were lowered from the normal 30°C level to 20°C (or at least lowered after ethanol concentrations became significant). This is necessary because of the inhibitory effect that high temperatures have on ethanol tolerance. The literature suggests that each of these alternatives should result in improvements in the ability of the yeast strain employed to produce ethanol. Only time will tell if such findings will be adopted, and, if so, if they are industrially significant.

Considerable research still remains to be done on the phenomenon of ethanol tolerance. Because the accumulation of intracellular ethanol has now been ruled out, a satisfactory answer (or mechanism) must be found to explain why conditions of high osmotic pressure have such deleterious effects on yeast ethanol tolerance. Is it primarily the effect of low water activity levels, with ethanol effects being secondary, or are the two connected in some synergistic manner? Likewise, it is still not clear why produced ethanol is so much more toxic to yeast than added ethanol. Since the conditions employed for these are different, the answer is likely to be found by analyzing the influence of each environmental and nutritional condition employed.

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